Investigating osmotic stress in mixed yeast cultures and its effects on wine composition

by

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Declaration

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Summary

Grape must gives rise to various stress conditions for the yeast inoculated for alcoholic fermentation. These include hyperosmotic stress due to the high initial sugar concentration and redox imbalances due to the fast depletion of oxygen. Under these stress conditions, *Saccharomyces cerevisiae* tends to produce glycerol as an osmoprotectant and to regenerate reducing equivalents. However, the production of glycerol often leads to increased acetic acid production. According to literature, it seems that many non-*Saccharomyces* yeasts have a different metabolic response to the above-mentioned stress conditions, especially since it has been found that they produce low levels of acetic acid. Only recently non-*Saccharomyces* yeasts were researched to be used as starter cultures in wine fermentations. It is found that they can confer beneficial characteristics to the resulting wine. However, most of the non-*Saccharomyces* yeasts lead to stuck fermentations as confirmed by this study. Therefore, if the positive characteristics of these yeasts were to be exploited in wine making they need to be inoculated together with *S. cerevisiae.* When two yeasts are inoculated together, they affect each other and consequently the wine.

In this context, the aim of this study was to investigate the metabolic response to hyperosmotic stress during wine fermentation of the following wine-related non-*Saccharomyces* yeasts: *Lachancea thermotolerans*, *Torulaspora delbrueckii* and *Starmerella bacillaris.* Fermentations were performed in a synthetic grape must medium with pure cultures of the mentioned strains as well as mixed cultures of each non-*Saccharomyces* yeast with *S. cerevisiae.* The fermentation behaviour was monitored and concentrations of various wine-related metabolites were determined. Concerning polyol concentrations, *S. cerevisiae* produced only glycerol while the non-*Saccharomyces* yeasts also produced other polyols. The low production of acetic acid in the non-*Saccharomyces* fermentations was confirmed especially in the case of *L. thermotolerans.* Moreover, this yeast produced high levels of the higher alcohols butanol and propanol. *St. bacillaris* produced significant levels of acetoin and isobutyric acid and *T. delbrueckii* produced an increased concentration of succinic acid. All these metabolites might play a role in maintaining intracellular redox balance. However, a more extensive systematic study is needed to investigate the extent of their involvement. The mixed cultures completed fermentation and had higher final glycerol levels than the control and lower acetic acid concentrations and therefore can contribute positively to the wine aroma. Furthermore, the mixed culture fermentations showed the potential of lowering the ethanol concentrations of wine.

Furthermore it has been shown in literature that the yeasts present in the mixed culture can affect each other on gene expression level as well. However, there is little genetic information available on non-*Saccharomyces* yeasts. In this study, we sequenced the genes involved in

glycerol and acetic acid biosynthesis of *L. thermotolerans* and *T. delbrueckii*. The gene sequences are fairly homologous with only a few differences. These gene sequences can be used to study gene expression of *GPD1* and *ALD6* from fermentation samples in order to determine to what extent the yeasts in a mixed culture influence the gene expression of one another.

Opsomming

Druiwemos gee oorsprong aan verskeie strestoestande vir die gis wat vir alkoholiese fermentasie geïnokuleer word. Hierdie strestoestande sluit hiper-osmotiese stres, as gevolg van die hoë suiker konsentrasie, in asook redoks wanbalanse toegeskryf aan die vinnige afname in beskikbare suurstof. Tydens hierdie toestande is *Saccharomyces cerevisiae* geneig om gliserol as beskerming teen die osmotiese stres te produseer, sowel as vir die regenereering van reduserings ekwivalente. Die produksie van gliserol lei egter dikwels tot toenemende asynsuur produksie. Volgens literatuur kom dit voor asof menige nie-*Saccharomyces* giste 'n ander metabolise reaksie tot die bogenoemde stresse het, omdat daar gevind is dat hulle laer vlakke van asynsuur produseer. Eers onlangs is navorsing gedoen op die potensiële gebruik van nie-*Saccharomyces* giste in gemengde kulture tydens wynfermentasies. Daar is bevind dat hulle voordelige eienskappe aan die wyn kan verleen. Meeste van die nie-*Saccharomyces* giste lei egter tot onvolledige fermentasies soos bevesting deur hierdie studie. Dus, indien die positiewe eienskappe van hierdie giste sou benut word in wynmaak sal hulle saam met *S. cerevisiae* geïnokuleer moet word. Wanneer twee giste saam geïnokuleer word, beïnvloed hulle mekaar en gevolglik die wyn.

In hierdie konteks was die doel van die betrokke studie om die metaboliese reaksie tot hiperosmotiese stress tydens wynfermentasies te ondersoek in die volgende wyn verwante nie-*Saccharomyces* giste: *Lachancea thermotolerans*, *Torulaspora delbrueckii* en *Starmerella bacillaris.* Fermentasies was in sintetiese druiwemos medium uitgevoer met rein kulture van die genoemde gisrasse, sowel as gemengde kulture van elke *nie-Saccharomyces* gis met *S. cerevisiae*. Die fermentasiegedarg is gemonitor en die konsentrasies van verskeie wyn verwante metaboliete is bepaal. Wat die poliol konsentrasies betref, het *S. cerevisiae* slegs gliserol geproduseer terwyl die nie-*Saccharomyces* giste additionele poliole ook geproduseer het. Die lae produksie van asynsuur in die nie-*Saccharomyces* fermentasies is bevestig, veral in die geval van *L. thermotolerans*. Verder produseer hierdie gis hoë vlakke van asetoïen en iso-bottersuur en *T. delbrueckii* produseer 'n hoër konsentrasie van suksiensuur. Al hierdie metaboliete mag 'n rol speel in die handhawing van intrasellulêre redoksbalans. 'n Meer uitgebreide, sistematiese studie is egter nodig om die mate van hul betrokkenheid te ondersoek. Die gemengde kulture het hul fermentasies voltooi en het hoër finale gliserol vlakke as die kontrole gehad, asook laer asynsuur konsentrasies en kan dus positief bydra tot die wyn aroma. Verder het die gemengde kultuur fermentasies die potensiaal om die etanol vlakke van wyn te verlaag, getoon.

Daar is verder in die literatuur gevind dat die giste teenwoordig in die gemengde kultuur mekaar op geenuitdrukkings vlak ook kan beïnvloed. Daar is egter min genetiese inligting beskikbaar vir die nie-*Saccharomyces* giste. In hierdie studie het ons die gene betrokke by die produksie van gliserol en asynsuur van *L. thermotolerans* en *T. delbrueckii* se nukleotied volgordes bepaal.

Die gevolglike nukleotied volgordes is redelik homoloog met net 'n paar verskille. Hierdie volgordes kan gebruik word om die geenuitdrukking van *GPD1* en *ALD6* vanaf fermentasie monsters te bestudeer om sodoende te bepaal tot watter mate die giste in 'n gemengde kultuur mekaar se geenuitdukking kan beïnvloed.

Biographical sketch

Marli Christel de Kock was born on 23 May 1990 in Cape Town and raised in Langebaan. She matriculated from Vredenburg High School in 2008 and in 2011 obtained a BSc-degree (Molecular Biology and Biotechnology) from Stellenbosch University. In 2012, Marli obtained a Hons-BSc degree in Wine Biotechnology and commenced with a MSc in Wine Biotechnology at the same university.

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Preface

This thesis is presented as a compilation of 4 chapters.

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Chapter 1

General introduction and project aims

Chapter 1: General introduction and project aims

1.1 Introduction

Fermentation of grape must to wine is a complex process in which yeasts play an essential role. The fermentation environment gives rise to various stress conditions such as hyperosmotic stress due to high initial sugar concentration and intracellular redox imbalance due to little or no oxygen. *Saccharomyces cerevisiae* is the main wine yeast conducting alcoholic fermentation. In order to counteract the impact of osmotic stress and to maintain redox balance in fermentative conditions, this yeast mainly synthesizes glycerol as an osmoprotectant and to regenerate reducing equivalents (Albertyn et al. 1994, Norbeck et al. 1996).

Although glycerol is the main polyol produced by yeasts to counteract the effects of hyperosmotic stress, there are reports of the production of other polyols (e.g. arabitol, mannitol, xylitol, erythritol) in addition to glycerol (Tokuoka et al. 1992, van Eck et al. 1993). Such responses were especially observed for non-*Saccharomyces* yeasts. Extensive research into the specific osmotic stress responses of *S. cerevisiae* and several osmotolerant yeast species have previously been conducted (Nevoight and Stahl 1997, Rep et al. 2000, Hohmann 2002, Michán et al. 2012, Dakal et al. 2014). However, not much research has been performed on wine-related non-*Saccharomyces* yeasts, even though they too have to survive the initial high sugar concentration of grape must including very high sugar musts such as those used to produce ice and botrytised wines. As mentioned above, *S. cerevisiae* also produces glycerol to maintain redox balance in fermentative conditions. It is not known whether it is the case for non-*Saccharomyces* wine yeasts as well. The production of higher alcohols can also assist in regeneration of NAD^+. Furthermore, it has been previously reported that a glycerol-deficient strain of *S. cerevisiae* produced increased amounts of certain higher alcohols (Jain et al. 2012). This could well be the case for non-*Saccharomyces* yeasts as well and it can impact the resulting wine if these yeasts were to be utilised.

The excess production of glycerol in response to osmotic stress and anaerobiosis often leads to increased acetic acid production in *S. cerevisiae*. Indeed, acetic acid is produced to reduce the NAD^+ generated during glycerol formation (Remize et al. 1999, de Barros Lopes et al. 2000). The elevated concentrations of acetic acid lead to an increase in volatile acidity which may be detrimental to wine quality (Pigeau and Inglis 2007). However, in various non-*Saccharomyces* yeasts the production of glycerol appears not to be linked to acetic acid production as observed in *S. cerevisiae*. For instance, *Starmerella bacillaris* (formerly known as *Candida zemplinina*) is known to produce elevated levels of glycerol, but relatively low levels of acetic acid under winemaking conditions (Ciani and Maccarelli 1998). Furthermore, it was found that no significant relationship between glycerol and acetic acid production exists in *Torulaspora delbrueckii* (Renault et al. 2009). *St. bacillaris*, *T. delbrueckii* and *Lachancea thermotolerans* are

consistent producers of low levels of acetic acid that do not rise under osmotic stress (Ciani and Maccarelli 1998, Kapsopoulou et al. 2005). These findings suggest that these non-*Saccharomyces* yeasts have developed other metabolic responses than *S. cerevisiae* to maintain redox balance when glycerol is produced in high amounts. In a study conducted in mutants of *S. cerevisiae* in which the *ALD6* gene responsible for acetic acid production was deleted under conditions where glycerol was overproduced, an increase in various compounds such as succinic acid, acetoin and 2,3-butanediol was observed (Cambon et al. 2006). The non-*Saccharomyces* yeasts might produce these compounds in high amounts to maintain redox balance and consequently it could affect the wine quality and aroma.

Although many non-*Saccharomyces* yeasts produce low amounts of acetic acid, they do not ferment as well as *S. cerevisiae* and often lead to stuck fermentations (Ciani et al. 2010). Therefore, in order to utilize the aforementioned characteristics of the non-*Saccharomyces* yeasts in terms of glycerol and acetic acid production and to have efficient fermentation rates in wine fermentations, studies were conducted on the use of these yeasts in mixed starter cultures together with *S. cerevisiae* strains (Romano et al. 2003, Ciani et al. 2006). The data suggest that such non-*Saccharomyces* yeasts in mixed cultures indeed tend to decrease levels of acetic acid compared to *S. cerevisiae* pure cultures (Ciani et al. 2006, Comitini et al. 2011). When co-inoculation with *T. delbrueckii* was investigated, it was observed that the glycerol production of the mixed culture was similar to that in *S. cerevisiae* pure culture, but the acetic acid concentration was lower (Bely et al. 2008). A similar observation was made when *L. thermotolerans* was used in a mixed culture fermentation (Comitini et al. 2011). Another example is a co-inoculation with *St. bacillaris* where significantly high amounts of glycerol are produced accompanied with low levels of acetic acid (Rantsiou et al. 2012).

The different yeasts, when inoculated together, interact with each other and this inoculation strategy impacts the glycerol and acetic acid levels in the resulting wine. However, exactly how the yeasts interact is largely unknown. Furthermore, little data exist on how one yeast in a mixed culture affects the gene expression of another. The presence of *St. bombicola* and *Metschnikowia pulcherrima* respectively in mixed culture fermentations with *S. cerevisiae* indeed has an impact on the gene expression of selected genes within *S. cerevisiae* (Milanovic et al. 2012, Sadoudi et al. 2014).

1.2 Rationale and aims of this project

We aimed to investigate the metabolic response to hyperosmotic stress during wine fermentation of the following selected non-*Saccharomyces* yeasts: *L. thermotolerans*, *T. delbrueckii* and *St. bacillaris.* Furthermore, the effects of the interaction in mixed cultures on wine composition were investigated in terms of differences in metabolite production compared to pure culture fermentations. The genetic data made recently available (i.e. genome sequences

of *L. thermotolerans* and *T. delbrueckii*) was exploited as far as possible in order to quantify the expression of genes involved in the glycerol and acetic acid biosynthesis.

In order to achieve this aim, three specific objectives were set:

1. To monitor the fermentation behaviour of the yeasts as pure or mixed starter cultures.
2. To determine the concentrations of additional or alternative compatible solutes.
3. To investigate the production of glycerol and acetic acid on a molecular level in terms of the gene expression of *GPD1* and *ALD6.*

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Chapter 2

Literature review

Glycerol and acetic acid production in yeast as response to hyperosmotic stress and redox imbalance in wine fermentations

Chapter 2: Glycerol and acetic acid production in yeast as response to hyperosmotic stress and redox imbalance in wine fermentations

2.1 Introduction

Yeasts are exposed to modifications in their natural environment to which they need to adapt in order to survive. These alterations include changes in the external solute concentration (osmolarity). A yeast cell experiences osmotic stress when a change in osmolarity occurs. Two kinds of osmotic stress exist: hyper- and hypo-osmotic. The former is caused by a higher solute concentration in the surrounding environment than inside the cell, while the latter is experienced when a decrease in extracellular osmolarity occurs. Examples of situations of osmotic stress include flooding or drought, ripening of fruits and food and beverages high in salt or sugar. This review will specifically focus on hyperosmotic stress in grape must since the fermenting yeast is inoculated into a high sugar medium that leads to an increase in extracellular osmolarity thereby creating a stressful environment for the yeast (Nevoight and Stahl 1997).

When the osmolarity of the extracellular environment increases, the surrounding water becomes less available for the cell. Consequently, a water efflux occurs, as water tends to flow from a compartment with low osmolarity to one with higher osmolarity (Tamas and Hohmann 2003). Therefore, if a yeast is in an environment with high osmolarity, water flows from the cell into the extracellular medium. The water efflux impacts the cell in various ways as will be discussed in the next section. Fortunately, yeasts have regulatory mechanisms in place to counteract the effects of osmotic stress by balancing the osmotic pressure inside the cell to the extracellular medium. It is achieved through the production of compatible solutes, which will also be discussed in this review. The cell cannot handle indefinite stress and very high osmotic pressure leads to growth arrest and cell death.

The osmotic stress response of yeast used as starter cultures in wine fermentations is of importance, as it ensures the survival of the yeast during fermentation. Furthermore, the response leads to the production of compatible solutes that affect wine composition. The production of these compounds is also important in redox balance since it involves a dehydrogenase reaction. Therefore, in order to maintain the redox balance during osmotic stress, the yeast produces other compounds, such as acetic acid, higher alcohols and fatty acids which impact the wine composition as well. Although the production of these metabolites are involved in other metabolic functions, they do have a role in redox balance. For the reasons mentioned above, redox balance during osmotic stress will be discussed in the review. Finally, strategies to lower acetic acid during wine fermentation will be reviewed.

2.2 Osmotic stress in yeast

As mentioned above, a yeast experiences osmotic stress when a change in the extracellular osmolarity occurs. Different yeasts can tolerate different osmotic pressures in the surrounding medium. Therefore, some yeasts are more osmotolerant than others (van Eck et al. 1993). Hyperosmotic stress leads to growth arrest of the cell due to either the loss in cell volume or turgor pressure that can eventually cause the cell to die under extreme osmotic pressure (Blomberg 2000). Morris et al. (1983) indeed observed a loss in viability of cells exposed to osmotic stress.

2.2.1 Cellular impact of osmotic stress

When a yeast cell is in an environment with increased osmolarity, water rapidly starts to flow from the cell. Consequently, the osmotic gradient across the plasma membrane drops. The water efflux impacts the cell in various ways such as a loss in turgor pressure that leads to a reduction in cell volume (Hohmann 2002). Cell shrinkage upon osmotic stress is not only reported for the model yeast, *Saccharomyces cerevisiae*, but also in other yeast species including *Zygosaccharomyces rouxii* (Morris et al. 1983, van Zyl et al. 1993). However, the cell partially recovers due to its implementation of a specific osmotic stress response (van Zyl et al. 1993).

Furthermore, the decrease in cell volume leads to changes in the plasma membrane regarding composition and structure with consequences on the permeability and fluidity. The membrane pulls on the cell wall after which the wall contracts (Dupont et al. 2011). Moreover, the permeability of the membrane increases. The loss of plasma membrane integrity leads to the leaking out of cellular content and that is thought to explain cell death occurring after osmotic shock by Dupont et al. (2011). In order to counteract this, the sterol production, especially that of ergosterol, increases (Hosono 1992, Wood et al. 1999, Dupont et al. 2011). Furthermore, Hosono (1992) also observed a decrease in phospholipids. The fact that the yeast aims to decrease the membrane permeability might be to retain glycerol or other compatible solutes inside (Hosono 1992). Rep et al. (2000) indeed reported changes in the expression of genes involved in lipid metabolism which could lead to the changes observed in the plasma membrane. Some authors have also hypothesized that the effect of osmotic pressure on the membrane could affect the activity and localization of various transmembrane proteins (Tamas and Hohmann 2003).

Increased osmolarity not only affects cell volume and the plasma membrane, but also the cytoskeleton. The change in the osmotic gradient across the membrane acts as a stimulus for the reversible rearrangement of actin filaments during osmotic stress. The actin filaments direct growth during budding to the emerging bud. Therefore, it is important that the

cytoskeleton gets reassembled in order for the cell to continue dividing. This indeed occurs through an actin-binding protein, Rah3 (Chowdhury et al. 1992, Logothetis et al. 2007).

In order to maintain viability, the cell has to counteract these osmotic stress effects and that is achieved through a response phenomenon known as osmoregulation. Consequently, the cell can recover and adapt, depending on the time period of the stress and the yeast species.

2.2.2 Osmoregulation

The aim of osmoregulation for the cell, according to Nevoight and Stahl (1997), is to maintain its general structure in terms of turgor pressure and volume, as well as to remain metabolically active in a medium with high osmolarity. The general response of *S. cerevisiae* to osmotic stress is shown in Fig. 1. The cell senses the change in osmolarity and sends a signal to the nucleus to enhance expression of genes involved in osmolyte synthesis.

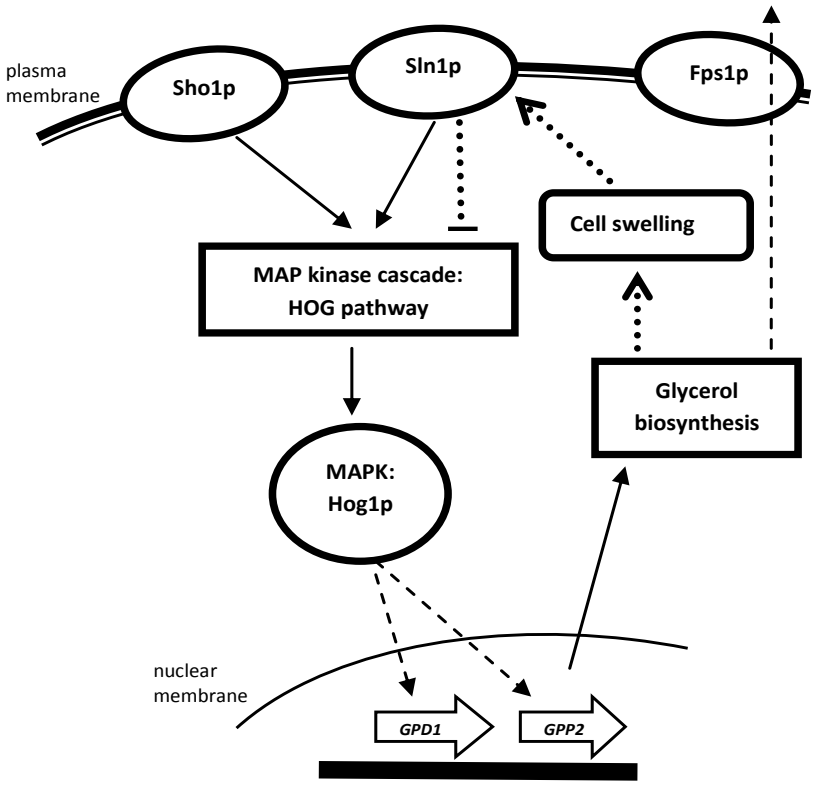

Fig. 1 Process of osmoregulation in *S. cerevisiae*. Adapted from Hohmann (2002) and Nevoigt and Stahl (1997).

The yeast recognises the osmotic pressure via two transmembrane proteins that act as osmosensors (Sln1p and Sho1p) (Maeda et al. 1995, Posas and Saito 1997). These osmosensors perceive the changes in the cell due to the water efflux and its various effects on the cell (as mentioned above). Subsequently, the signal is relayed through a MAP kinase signal transduction pathway known as the High Osmolarity Glycerol (HOG) pathway. The MAP kinase,

Hog1p, is activated (through phosphorylation) by this pathway and then transferred to the nucleus where it leads to transcriptional responses. Induction of the expression of *GPD1*, *GPP2* and *ALD6* amongst other genes is regulated by the HOG pathway (Nevoigt and Stahl 1997). The mentioned genes encode enzymes responsible for glycerol and acetic acid production in yeast under osmotic stress conditions.

Signalling leads to the production of one or more compatible solutes (also known as osmoprotectants or osmolytes). The accumulation of these compounds eventually leads to cell swelling (effect on turgor pressure) which in turn activates the sensor Sln1p. This leads to the deactivation of the HOG pathway (Tao et al. 1999). When the osmotic stress is alleviated, the accumulated solute is excreted via a membrane transporter, Fsp1p, in *S. cerevisiae* (Fig. 1). Indeed, in a study conducted by Kayingo et al. (2001), it was shown that upon hypo-osmotic shock following hyperosmotic stress, a decrease in the intracellular compatible solute levels correspond to an increase in its external concentration. The authors suggest that the yeast mainly releases the accumulated compatible solute and do not metabolise it.

Although the HOG pathway has been mostly studied in *S. cerevisiae*, it is not only functional in this species. Components of this pathway have been identified in other yeast species (Hohmann 2002) namely, *Candida albicans* (Alonso-Monge et al. 1999, Calera and Calderone 1999), *Z. rouxii* (Iwaki et al. 1999, Dakal et al. 2014), *Debaryomyces hansenii* (Bansal and Mondal 2000), *Candida utilis* and *Kluyveromyces lactis* (Siderius et al. 2000).

The production and accumulation of one or more compatible solutes in the cell counteract the outflow of water and help to balance the intracellular osmotic pressure with that of the extracellular environment (Nevoigt and Stahl 1997). These solutes are qualified as compatible, because they can be accumulated in high concentrations in the cell without significant enzyme inhibition or inactivation (Brown 1976, 1978). These compounds are retained in the cell as long as the osmotic stress condition persists. As mentioned in the previous paragraph, the compatible solute is released from the cell when the osmotic pressure decreases. Brown (1974) demonstrated that the major difference between strongly and weakly osmotolerant yeasts resides in the property of the former to accumulate high concentrations of polyols, which act as compatible solutes.

It was found that the main compatible solute formed in yeast is glycerol (Nevoight and Stahl 1997). Other polyols have also been shown to exhibit osmoprotective abilities, but are not as responsive to osmotic stress as glycerol (van Eck et al. 1993). Accumulation of solutes such as betaines and amino acids has also been observed in bacteria and plants in response to osmotic stress, but van Eck et al. (1993) failed to find other compatibles solutes than polyols in yeasts. Table 1 lists examples of polyols that different yeasts accumulate under osmotic stress.

S. cerevisiae failed to produce other polyols than glycerol in a study performed by van Eck et al. (1993). In a study conducted by Tokuoka et al. (1992), seven yeast strains were evaluated to determine which polyols they produce when confronted with osmotic stress (high

glucose, sucrose and sodium chloride). All the yeasts accumulated glycerol initially, after which several of the non-*Saccharomyces* species produced other polyols such as arabitol and erythritol (Table 1). Another study investigated which compatible solutes are released after a hypo-osmotic shock and the authors also observed that arabitol and erythritol were involved in *Z. rouxii* and *Pichia sorbitophila* respectively (Kayingo et al. 2001). van Eck et al. (1993) conducted their experiments in high sugar and high salt media and found that mainly glycerol was produced, but the production of arabitol and mannitol was also observed. Interestingly, more polyols were produced in the medium with high sugar than high salt. A study by Shen et al. (1999) engineered a *S. cerevisiae* strain deficient in glycerol biosynthesis genes to produce sorbitol and mannitol. It was found that these polyols do protect the cell during osmotic stress, but not as efficiently as glycerol at the same concentrations.

Table 1 Polyols produced by different yeast species during osmotic stress.

Species	Polyol produced as compatible solute						Reference
	Glycerol	Arabitol	Mannitol	Erythritol	Xylitol	Ribitol	
Saccharomyces cerevisiae	✓						
Torulaspora delbrueckii	✓	✓					Lucca et al. 2002, Tokuoka et al. 1992
Zygosaccharomyces rouxii	✓	✓	✓				Tokuoka et al. 1992, Groleau et al. 1995
Hansenula anomala	✓	✓					Bellinger et al. 1988, Tokuoka et al. 1992
Debaryomyces hansenii	✓	✓			✓		Tokuoka et al. 1992, Koganti et al. 2011
Candida tropicalis	✓	✓					Tokuoka et al. 1992
Candida magnoliae	✓		✓	✓			van Eck et al. 1993, Yu et al. 2006
Candida albicans	✓	✓				✓	Phyffer and Rast 1989, Kayingo and Wong 2005
Pichia sorbitophila	✓	✓		✓			Tokuoka et al. 1992, Kayingo et al. 2001
Trichosporonoides megachiliensis	✓			✓			Kobayashi et al. 2012

The disaccharide trehalose has also been shown to be produced during osmotic stress conditions in yeast (MacKenzie et al. 1988). The protective ability of trehalose lies mainly in its ability to stabilise proteins (Singer et al. 1998, Blomberg 2000). However, it seems that this compound is produced under several stress conditions, rendering it a more general stress protectant. It is not clear whether this disaccharide specifically acts as an osmolyte in yeast as it does in bacteria (Hohmann 2002). However, glycerol remains the most common polyol to be synthesised as compatible solute during osmotic stress in yeast.

2.2.3 Production of glycerol as osmoprotectant

The mechanism of osmoregulation in yeast is based on adjusting the intracellular glycerol concentrations in accordance to the osmolarity of the extracellular environment (Norbeck et al. 1996). The intracellular glycerol levels are determined by its formation, retention or accumulation, catabolism and transport in and out of the cell (Nevoigt and Stahl 1997, Remize et al. 2001).

However, increased glycerol levels in a cell subjected to high osmolarity are mostly due to increased production of this polyol in the cell. This is a consequence of the carbon metabolic flux that is directed towards glycerol at the expense of ethanol production (Nevoigt and Stahl 1997). It correlates with an observed decrease in rate of alcohol dehydrogenase synthesis (Blomberg 1995).

2.2.3.1 Glycerol biosynthesis

Glycerol is produced in two enzymatic steps as part of the central carbon metabolism in yeast (Fig. 3). Firstly, dihydroxyacetone phosphate (formed in the glycolysis pathway from glucose) is converted to glycerol-3-phosphate via NADH-dependent glycerol-3-phosphate dehydrogenases. Subsequently, glycerol-3-phosphate is dephosphorylated by glycerol-3-phosphatases to form glycerol (Scanes et al. 1998).

Two gene families are involved in the glycerol biosynthesis pathway in yeast (Table 2). Both families consist of two genes each, though it is not necessarily the case for all yeasts. The first family encodes the glycerol-3-phosphate dehydrogenases. *GPD1* and *GPD2* are highly homologous and both lead to the formation of glycerol. However, their expression is induced under different environmental conditions (Albertyn et al. 1994, Ansell et al. 1997). The expression of *GPD1* is induced under osmotic stress conditions (Albertyn et al. 1994, Ansell et al. 1997, Remize et al. 2001). This seems to be the case for salt as well as sugar stress (Du et al. 2012). *GPD2* expression is induced under semi-anaerobic to anaerobic conditions, which indicates that the expression of this gene is under redox control (Albertyn et al. 1994, Ansell et al. 1997, Remize et al. 2001). Furthermore, it has been reported that *GPD1* can partially substitute for *GPD2* (Ansell et al. 1997).

The glycerol-3-phosphatases are encoded by the genes *GPP1* and *GPP2*, which are also highly homologous and can substitute for each other (Pahlman et al. 2001). Overexpression studies of these two genes showed that they do not significantly promote the formation of glycerol, which indicates that this step is not rate limiting in glycerol biosynthesis (Remize et al. 2001, Pahlman et al. 2001).

Norbeck and Blomberg (1997) reported the upregulation of genes responsible for glycerol catabolism via the dihydroxyacetone pathway during salt stress. This could provide an overflow path for fine-tuning glycerol levels during stress together with the glycerol transporter,

Fps1p. Also, this catabolic pathway for glycerol could act as a transhydrogenase to convert NADH to NADPH.

Table 2 Characteristics of gene families operative in the glycerol biosynthesis (*Saccharomyces* genome database: www.yeastgenome.org).

Gene	Alias	Enzyme	Enzyme function/pathway	Cell compartment	Additional information
GPD1	*HOR1*	Glycerol-3-phosphate dehydrogenase	Glycerol Biosynthesis Converts DHAP to GL3-P	Cytosol	Co-factor: NAD^+ Main enzyme for glycerol synthesis
GPD2	*GPD3*	Glycerol-3-phosphate dehydrogenase	Glycerol Biosynthesis Converts DHAP to GL3-P	Cytosol	Co-factor: NAD^+
GPP1	*RHR2*	DL-glycerol-3-phosphatase	Glycerol Biosynthesis Converts GL3-P to glycerol	Cytosol	
GPP2	*HOR2*	DL-glycerol-3-phosphatase	Glycerol Biosynthesis Converts GL3-P to glycerol	Cytosol	

To summarise, during osmotic stress, the expression of *GPD1* and *GPP2* is induced and during anaerobic conditions the expression of *GPD2* and *GPP1* is enhanced (Remize et al. 2001, Hohmann 2002, Biyela 2008).

2.2.3.2 Glycerol uptake

Glycerol movement across the plasma membrane occurs via passive diffusion or active facilitated diffusion transport via Fps1p in *S. cerevisiae*. However, Fps1p restricts the efflux of glycerol during osmotic stress conditions, although it is not exactly known how this protein functions and senses osmotic stress. This transporter is mainly responsible for rapid release of glycerol during hypo-osmotic stress conditions (Toh et al. 2001). Furthermore, Fps1p facilitates glycerol uptake (Luyten et al. 1995).

S. cerevisiae can also take up glycerol through electrogenic proton symport facilitated by membrane proteins Gup1 and 2 when it is deficient in glycerol biosynthesis (e.g. *gpd1Δ* mutant) or grown on glycerol (Holst et al. 2000). It has been reported that a few other yeast species have an active glycerol uptake system; they are mostly osmotolerant yeasts such as *D. hansenii* and *P. sorbitophila* (Lages et al. 1999). Differences between strains regarding the active uptake of glycerol during osmotic stress may occur. Indeed, although van Zyl et al. (1990) reported that *Z. rouxii* possesses an active sodium-driven glycerol transport system, Lages et al. (1999) could not find such a transporter in this species. According to the former authors, it allows this species to take up glycerol and accumulate it intracellularly.

2.2.3.3 Additional functions

Glycerol is not only produced as osmoprotectant in yeast, but also has additional functions. The glycerol metabolic pathway is involved in phospholipid biosynthesis. Phospholipids indeed consist of a glycerol backbone esterified with fatty acids and a phosphate group (Daum et al. 1998).

Furthermore, this polyol acts as a redox sink when the yeast needs to survive under anaerobic conditions such as during alcoholic fermentation (Norbeck et al. 1996). Under such conditions, the NADH produced in biosynthetic reactions cannot be oxidised by the electron transport chain in the mitochondria. Subsequently, an endogenous electron acceptor is required and such an acceptor is provided in the formation of glycerol (Ansell et al. 1997, Bakker et al. 2001).

Redox balance under fermentative conditions and osmotic stress will be discussed further in the next section.

2.3 Alcoholic fermentation: osmotic stress and redox balance

During alcoholic fermentation of grape must, the yeast needs to survive under various stress conditions including osmotic stress (discussed above) and anaerobiosis. Thus, mechanisms to maintain redox balance should be available in order for the yeast to remain metabolically active since most metabolic reactions in the cell involve oxidation and reduction. Redox balance is known as the balance between oxidative and reductive equivalents.

The ratio between the pyridine nucleotides in the two co-enzyme systems (redox couple) $NADH/NAD^+$ and $NADPH/NADP^+$ is essential for the intracellular redox balance. In other words, reduction of NAD^+ should be on par with reoxidation of NADH. NADPH is generally used in assimilatory reactions. Its role is limited in fermentative sugar metabolism, although NADPH-dependent acetate production should not be overlooked (Bakker et al. 2001).

In the presence of oxygen, the yeast follows a respiratory metabolism. However, in the case of Crabtree positive yeasts, such as *S. cerevisiae*, if the sugar concentration is high, the yeast will ferment even in the presence of oxygen. Oxidation of the substrate, such as hexose sugars leads to the production of energy through oxidative phosphorylation in the electron transport chain, where oxygen serves as the electron acceptor. Consequently, a proton-motive force is established over the mitochondrial membrane that drives the energy requiring processes in the cell (Ansell et al. 1997). This proton-motive force is established through the re-oxidation of NADH by the electron transport chain. NADH cannot pass through biological membranes. Therefore, it has to be re-oxidised in the compartment where it was produced or be actively transported to another compartment. Consequently, the NADH produced in the cytosol has to be transported to the mitochondria as reviewed by Jain (2010).

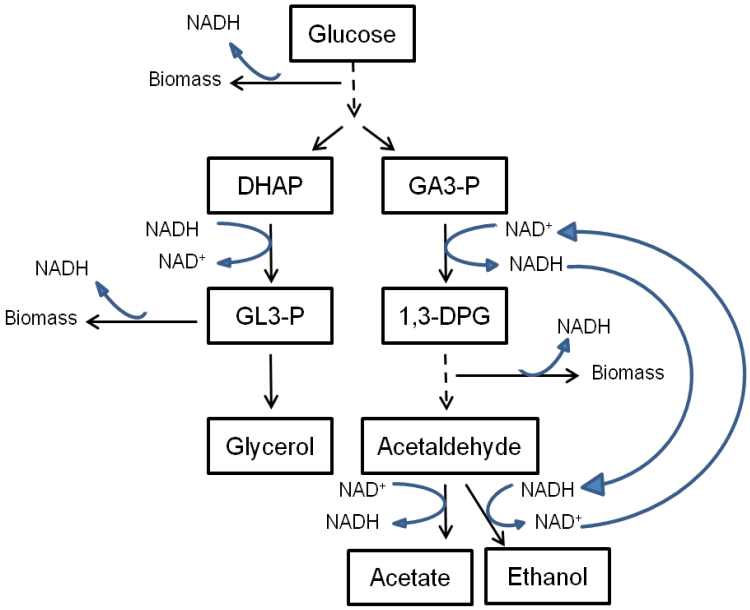

Fig. 2 Carbon metabolism and redox balance under fermentative growth for *S. cerevisiae* (Adapted from Jain 2010).

However, under fermentative conditions, little to no oxygen is present to serve as acceptor in the electron transport chain. Consequently, energy for cell functioning is solely obtained from substrate level phosphorylation during glycolysis (Ansell et al. 1997). In terms of intracellular redox balance, the fermentation process is known to be redox neutral. This means that the NADH produced during glycolysis, is converted back to NAD^+ when acetaldehyde is reduced to ethanol (Fig. 3). Subsequently, the regenerated NAD^+ can be used in glycolysis again. Yeasts also do not have a transhydrogenase to convert NADH to NAD^+ or vice versa.

Besides the glycolytic pathway being a major source of NADH when the yeast grows on hexoses, a surplus of NADH is formed in biosynthetic reactions, especially during amino acid synthesis (Albers et al. 1996, Bakker 2001). Subsequently, the amino acids are involved in biomass formation and this process subsequently results in a net production of NADH (Bakker et al. 2001) (Fig. 3).

Therefore, under fermentative conditions, the yeasts have to rely on the production of a reduced metabolite to rid the cell of surplus NADH and regenerate NAD^+ (Pigeau and Inglis 2005, Jain 2010). Glycerol has been shown to be the main metabolite produced to maintain intracellular redox balance in fermentative conditions (Albertyn et al. 1994), but other compounds may also be involved, such as different polyols and higher alcohols.

2.3.1 Higher alcohols

Higher alcohols are mostly synthesised from amino acids via the Ehrlich pathway (Hazelwood et al. 2008). This pathway consists of three steps as shown in Fig. 4. Firstly, the amino acid is transaminated to the corresponding keto acid, then decarboxylated to the aldehyde. Subsequently, the aldehyde is reduced to the corresponding higher alcohol. It is during this final

step that NADH is reoxidised to NAD^+ (Hazelwood et al. 2008). In terms of redox balance, it has been hypothesized that the formation of higher alcohols during fermentative growth acts as a redox sink for reoxidation of surplus NADH (Schoondermark-Stolk et al. 2005, Hazelwood et al. 2008). The production of higher alcohols plays an important role in wine fermentations since they contribute to the aroma profile of the wine; moreover, they are precursors of acetate esters which are also sensorially important in wine.

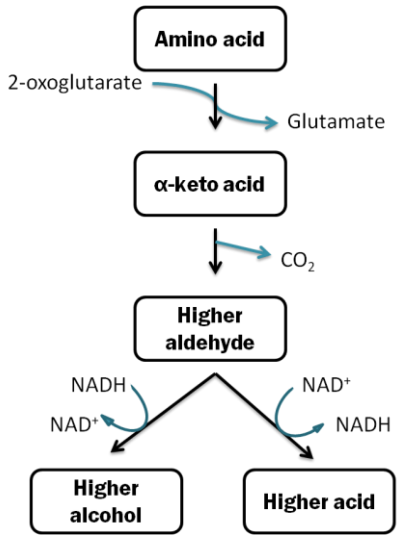

Fig. 3 Simplified Ehrlich pathway adapted from Hazelwood et al. (2008).

2.3.2 Glycerol

Glycerol is usually found in wine at concentrations ranging from 4 and 10 g/L (Scanes et al. 1998, de Barros Lopes et al. 2000). In high sugar fermentations, such as ice wine, the glycerol levels can increase up to about 12-17 g/L (Mills et al. 2002, Pigeau and Inglis 2007). This polyol does not directly impact the aroma profile of the wine as it is a non-volatile metabolite. It does, however, contribute to the mouthfeel and smoothness of the wine (Scanes et al. 1998).

As mentioned above, glycerol is produced to maintain redox balance in grape must fermentation in order to oxidize the NADH surplus formed during biomass production (Fig. 3). In addition, glycerol is produced as osmoprotectant. Therefore, during fermentation in a medium such as grape must, this polyol is produced in high amounts. The increased glycerol levels cause a redox imbalance that leads to the production of certain by-products (Bakker et al. 2001). It has indeed been reported that as a consequence of this increased synthesis of glycerol, an increase in certain metabolites including 2,3-butanediol, acetoin, acetaldehyde, acetic acid and succinate was observed (Remize et al. 1999, de Barros Lopes et al. 2000, Remize et al. 2001, Cambon et al. 2006). In *S. cerevisiae*, the most prominent increase is that of acetic acid (Erasmus et al. 2004).

The increase in acetic acid as a consequence of glycerol overproduction is even more prominent in high sugar fermentations such as botrytized or ice wines, because the glycerol concentration is also higher. It was observed that for *S. cerevisiae* the higher the initial sugar concentration is, the higher the resultant glycerol and acetic concentrations. The glycerol levels increase approximately 2-3 fold and that of acetic acid 3-6 fold when sugar concentrations are increased from approximately 200-360 g/L (Erasmus et al. 2004, Pigeau and Inglis 2007, Renault et al. 2009).

2.3.3 Acetic acid

Acetic acid is an organic acid formed as an intermediate in the pyruvate dehydrogenase by-pass (Fig. 5). This pathway is mainly responsible for providing acetyl-CoA for the cell and can take place in either the mitochondria or the cytosol (Saint-Prix et al. 2004).

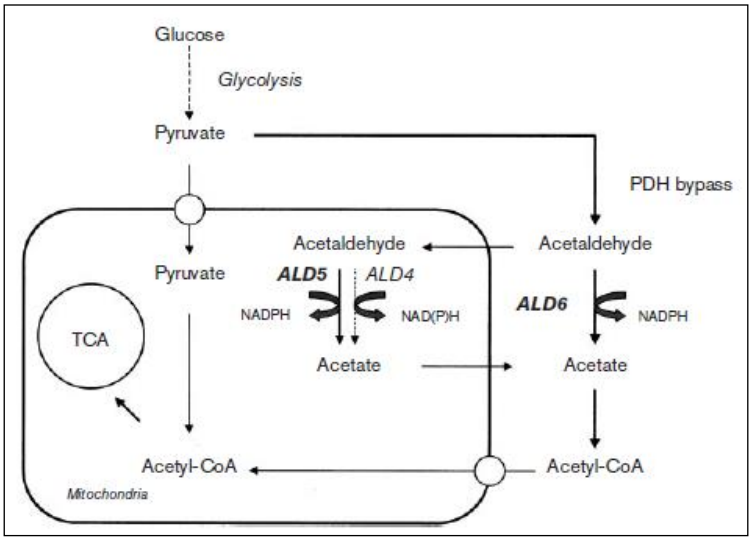

Fig. 4 Production of acetate via the PDH by-pass (Saint-Prix et al. 2004).

The enzymes involved in acetic acid formation in yeast are known as aldehyde dehydrogenases and are encoded by the family of genes listed in Table 3. Regarding wine fermentations, the aldehyde dehydrogenase encoded by *ALD6* is the main enzyme responsible for acetic acid formation (Cambon et al. 2006). However, *ALD3* may contribute to acetic acid formation in very high sugar fermentations, such as ice wine (Pigeau and Inglis 2005).

Acetic acid is the main component of volatile acidity in wine. It causes a vinegary aroma that is detrimental to the wine quality. It is usually associated with spoilage (Pigeau and Inglis 2007). The concentration of this metabolite is generally lower than 0.5 g/L in wine and should not exceed 1.2 g/L according to legislation (OIV 2009). Therefore, it would be beneficial for wine quality if the acetic acid concentrations were kept as low as possible.

Table 3 Aldehyde dehydrogenases involved in acetic acid production (*Saccharomyces* genome database: www.yeastgenome.org).

Gene	Alias	Enzyme function/pathway	Compartment in cell	Additional information
ALD6	*ALD1*	Converts acetaldehyde to acetate in the PDH bypass	Cytosol	Co-factors: Mg^{2+} and NADP
ALD2		Involved in ethanol oxidation. Involved in β-alanine synthesis.	Cytosol	Co-factor: NAD^+ Stress inducible
ALD3		Involved in ethanol oxidation. Involved in β-alanine synthesis.	Cytosol	Co-factor: NAD^+ Stress inducible
ALD4	*ALD7*	Converts acetaldehyde to acetate in the PDH by-pass.	Mitochondria	Co-factors: K^+ and NAD^+ or $NADP^+$
ALD5		Acetate formation. Synthesis of electron transport chain components.	Mitochondria	Co-factors: K^+ and $NADP^+$

2.4 The use of mixed cultures to reduce acetic acid levels in wine

Industrial methods have been developed to reduce volatile acidity of which acetic acid is the main component in wine. They are based on physicochemical principles and include reverse osmosis and anion exchange (Zoecklein et al. 1995, Vilela-Moura et al. 2011). However, only biological methods will be discussed in this review. The latter include refermentation of wines with high volatile acidity. This technique relies on the acetic acid consumption abilities of yeasts. Refermentation is performed by adding grape must to the finished wine. However, Vilela-Moura et al. (2010, 2013) reported that certain commercial strains of *S. cerevisiae* can successfully deacidify wine. In their trial, the acetic acid was reduced even further when the cells were immobilized in alginate-chitosan beads. For these strains to lower the volatile acidity, the wine needs to be stabilized at total SO_2 levels of 70 mg/L or lower. Refermentation may nevertheless have unexpected final results as it is not known which indigenous yeasts are present in the must and how the wine will be affected (Zoecklein et al. 1995).

Regarding winemaking processes, it was found that the time and amount of nitrogen added have an effect on the volatile acidity at the end of high sugar fermentations (Bely et al. 2003). Thus, it is not only the specific species or strain that has an effect, but also the must composition.

Furthermore, the acetic acid can be lowered directly during fermentation in an attempt to prevent the production of elevated levels. Research has shown that a strain of *S. cerevisiae* can be genetically altered to produce lower acetic acid concentrations. Cambon et al. (2006) deleted *ALD6* in a *GPD1* overexpressing strain and found that it effectively reduces the acetic acid levels. The same was observed when *ALD6* was deleted in a *GPD2* overexpressing strain by Eglinton et al. (2002). However, it was also found that the deletion of *ALD6* leads to the formation of various by-products which might be detrimental to the wine quality as in the case of acetic acid (Remize et al. 2000).

Many non-*Saccharomyces* wine yeasts are reported to produce less acetic acid than *S. cerevisiae*. Until recently, these yeasts naturally occurring in fermenting musts were often regarded as spoilage micro-organisms in the winemaking process (Ciani et al. 2010, Ciani and Comitini 2011). Furthermore, most of these yeasts show limited fermentation aptitudes which can result in stuck fermentations. As a result, *S. cerevisiae* is commonly used as a starter culture (Pretorius 2000) in order to deliver a reliable product. However, after further research, these yeasts have proved to have great significance for the winemaking industry, since they represent a poorly explored biodiversity (Comitini et al. 2011). The use of indigenous strains may indeed assure the maintenance of typical sensory properties of wines from a given geographic region (Callejon et al. 2010), as well as enhance quality, improve complexity and modify undesired factors in the wine (Comitini et al. 2011).

Acetic acid is one such undesirable factor that can be lowered by these yeasts. Indeed, non-*Saccharomyces* yeasts do not necessarily produce increased levels of acetic acid when high concentrations of glycerol are produced. It was observed that in *T. delbrueckii,* there is no significant relationship between glycerol and acetic acid productions (Renault et al. 2009). Furthermore, although *Starmerella bacillaris* (formerly known as *Candida zemplinina*) is known to produce elevated levels of glycerol, this yeast synthesises low levels of acetic acid under winemaking conditions (Ciani and Maccarelli 1998). The same was observed with a certain strain of *Hanseniaspora uvarum* by de Benedictis et al. (2011). *Lachancea themotolerans* and *Torulaspora delbrueckii* similarly produce lower levels of acetic acid than *S. cerevisiae* (Comitini et al. 2011). However, the level of production of acetic acid depends on the strain within a specific species of non-*Saccharomyces* yeast. Ciani and Maccarelli (1998) indeed reported that *H. uvarum* produces high levels of acetate in contrast to what de Benedictis et al. (2011) found. Also, certain strains of *T. delbrueckii* produce even more acetate than *S. cerevisiae* (Renault et al. 2009). It is not known why these yeasts produce low acetic acid levels. It can only be hypothesized that non-*Saccharomyces* yeasts have alternative ways to maintain redox balance during osmotic stress.

In order to utilize the aforementioned characteristics of the non-*Saccharomyces* yeasts and to have efficient fermentation rates in wine fermentation, studies were conducted on the use of these yeasts in mixed cultures with *S. cerevisiae* (Refer to Table 4 for examples). It was shown that mixed culture fermentations with *L. thermotolerans*, *Metschnikowia pulcherrima*, *T. delbrueckii* and *Pichia fermentans* lead to greater or similar glycerol levels and reduced acetic acid levels in comparison to *S. cerevisiae* pure cultures (Table 4) (Clemente-Jimnez et al. 2005, Comitini et al. 2011). Another example of mixed cultures for reducing acetic acid in sweet wine fermentations is the use of *St. bacillaris*. As mentioned above, this yeast produces significantly higher amounts of glycerol and lower levels of acetic acid than *S. cerevisiae*. *St. bacillaris* is osmotolerant and fructophilic and might be able to utilize the sugar at the beginning of

fermentation and thus lower the sugars that would lead to osmotic stress for *S. cerevisiae* and consequent increased acetic acid levels (Rantsiou et al. 2012).

Table 4 Effect on acetic acid and glycerol production in non-*Saccharomyces* and in mixed cultures with *S. cerevisiae*.

Non-*Saccharomyces* species	Pure culture	Mixed culture with *S. cerevisiae* [a]	Reference studies
T. delbrueckii	Low acetate	Reduced acetate levels	Renault et al. 2009, Comitini et al. 2011
C. zemplinina	High glycerol	Increase in glycerol levels Reduced acetic acid	Rantsiou et al. 2012
L. thermotolerans	Low acetate	Reduced acetate levels	Comitini et al. 2011
P. fermentans	Low acetate	Reduced or similar acetate levels	Clemente-Jimnez et al. 2005
M. pulcherrima	Low acetate	Reduced acetate Increased glycerol	Comitini et al. 2011

[a] Effects are compared with *S. cerevisiae* pure cultures.

It should be kept in mind that in mixed cultures, the yeast species do not co-exist passively, but rather interact with one another in various ways (Charoenchai et al. 1997, Hansen et al. 2001, Fleet 2003, Nissen and Arneborg 2003, Cheraiti et al. 2005). These interactions can have positive or negative effects on the species and consequently the wine. The positive contributions of the non-*Saccharomyces* yeasts are highly dependent on the persistence of these yeasts in the fermentation. *S. cerevisiae* usually dominates wine fermentations, mainly because of its high tolerance to ethanol and oxygen limitation. It also depends on the strain combination and type of inoculation (co-inoculation or sequential).

A hypothesis for the lower acetic acid when non-*Saccharomyces* yeasts are inoculated together with *S. cerevisiae* is the uptake of acetic acid produced by *S. cerevisiae* by the non-*Saccharomyces* yeasts. For most strains of *S. cerevisiae*, the transport and metabolism of acetic acid is subjected to glucose repression, therefore, it uses the acetic acid only after the glucose is depleted (Vilela-Moura et al. 2011). A few other yeasts display similar behaviour: *Candida utilis* (Leão and van Uden 1986), *T. delbrueckii* (Casal and Leão 1995) and *Dekkera anomala* (Geros et al. 2000). However, evidence exists that certain yeasts can consume acetic acid together with glucose. Vilela-Moura et al. (2008) indeed reported this for a strain of *L. thermotolerans*, as well as three commercial strains of *S. cerevisiae* under limited-aerobic conditions. It has also been shown that *Zygosaccharomyces bailii* can consume acetic acid and glucose simultaneously (Sousa et al. 1998, Rodrigues et al. 2012). Although the potential of certain yeasts to consume glucose and acetic acid together exist, more research needs to be performed to screen wine yeasts for this characteristic.

2.5 Summary and future outlooks

Grape must is a high sugar environment with concentrations of approximately 140-260 g/L for table wines and 320-400 g/L for botrytized grapes or Ice wine must. Therefore, the wine yeasts experience osmotic stress when inoculated for alcoholic fermentation. The high osmotic pressure causes water to flow out of the cell and this affects the cell negatively. If the yeast does not counteract the impact of the pressure, it loses viability and dies off. Therefore, yeasts have mechanisms in place in order to survive the stress; they are collectively known as osmoregulation. The yeast perceives the stress by membrane receptors and physical changes in the membrane and cytoskeleton. Subsequently, the signal is relayed via the HOG pathway to the nucleus where the expression of certain genes is affected. The expression of genes responsible for glycerol production such as *GPD1* and *GPP2* is induced. Consequently, there is an increase in glycerol synthesis. Glycerol acts as a compatible solute to counteract the water efflux by increasing the solute levels in the cell.

Furthermore, glycerol is produced to maintain redox balance for growth in anaerobic conditions. Considering that the fermentation of grape must starts with high sugar and takes place anaerobically, increasing levels of glycerol are synthesised. As a result, *S. cerevisiae* produces increasing levels of acetic acid. This acid forms the major part of volatile acidity of finished wine and high levels are detrimental to the quality. However, it has been reported that certain non-*Saccharomyces* yeasts do not respond to increased glycerol concentrations with an increase in acetic acid. Therefore, these yeasts can be utilized together with *S. cerevisiae* in wine fermentations to lower acetic acid concentrations, especially in high sugar fermentations.

Recent studies have suggested that non-*Saccharomyces* yeasts indeed respond to osmotic stress and maintain redox balance differently than *S. cerevisiae*. However, it is not clear exactly how they respond to the stress and further research into the mechanisms is needed in order to fully optimise the utilization of non-*Saccharomyces* yeasts in wine fermentations. Fundamental research on how osmoregulation functions in non-*Saccharomyces* yeasts regarding osmosensors, pathways and compatible solutes needs to be performed. It would also be beneficial for the wine industry to know which polyols these yeasts produce as compatible solutes and in what concentrations, as they might have an effect on wine properties. Research into which additional metabolites these yeasts produce to maintain redox balance can be helpful in strain selection. Further research should also be performed in order to establish whether the non-*Saccharomyces* yeasts can utilize the acetic acid produced by *S. cerevisiae* especially under wine-making conditions.

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Chapter 3

Research results

Investigating osmotic stress in mixed yeast cultures and its effects on wine composition

Chapter 3: Research results - Investigating osmotic stress in mixed yeast cultures and its effects on wine composition

3.1 Introduction

Grape must is a non-sterile environment that hosts numerous microorganisms, in particular, various species of yeasts. These yeasts are typically divided into two groups, the *Saccharomyces* (especially *Saccharomyces cerevisiae*) and the non-*Saccharomyces* yeasts. Yeasts belonging to both groups can ferment the grape must to various extents and are indeed present in spontaneous fermentation that is carried out by a succession of different yeast species (Di Maro et al. 2007, Comitini et al. 2011). After three to four days, most of the non-*Saccharomyces* yeasts die off and *S. cerevisiae* starts to dominate (Di Maro et al. 2007, Comitini et al. 2011), since it is a stronger fermenter with tolerance to high ethanol levels (Bely et al. 2008).

Until recently, non-*Saccharomyces* yeasts in fermenting musts were often regarded as spoilage microorganisms in the winemaking process (Ciani et al. 2010, Ciani and Comitini 2011). Furthermore, most of these yeasts show limited fermentation aptitudes which can result in stuck fermentations. As a result, *S. cerevisiae* is commonly used as starter culture (Pretorius 2000) in order to deliver a reliable product. However, the non-*Saccharomyces* yeasts might prove to have greater significance than initially thought. The use of native strains may indeed assure the maintenance of typical sensory properties of wines from a given region (Callejon et al. 2010), as well as enhance quality, improve complexity and modify undesired factors in the wine (Comitini et al. 2011).

Fermentation in grape must gives rise to various stress conditions for the inoculated yeast. For instance, hyperosmotic stress due to high initial sugar concentration and also intracellular redox imbalance due to little or no oxygen. Under these stress conditions, *S. cerevisiae* tends to produce glycerol as a compatible solute and to regenerate reducing equivalents (Norbeck et al. 1996, Ansell et al. 1997). The genes involved in glycerol biosynthesis are regulated differently depending on the specific need for glycerol. The osmotic stress response includes the induction of *GPD1* and *GPP2* expression and the unavailability of oxygen induces expression of *GPD2* and *GPP1* (Remize et al. 2001, Biyela 2008).

In literature, lower levels of glycerol production have been reported for *Lachancea thermotolerans* and *Torulaspora delbrueckii* compared to *S. cerevisiae* (Ciani and Maccarelli 1998, Kapsopoulou et al. 2005, Renault et al. 2009). *Starmerella bacillaris* on the other hand is known for its high glycerol production (Tofalo et al. 2012). In *S. cerevisiae*, increased levels of glycerol subsequently lead to an increase in acetic acid production in order to maintain redox balance (Remize et al. 1999, Erasmus et al. 2004). The enhanced acetic acid concentration is mainly the consequence of increased *ALD6* expression (Remize et al. 2000, Cambon et al.

2006). This phenomenon is however not observed as consistently in non-*Saccharomyces* yeasts. For instance, *St. bacillaris* is known to produce elevated levels of glycerol, but relatively low levels of acetic acid under winemaking conditions (Ciani and Maccarelli 1998). *L. themotolerans* and *T. delbrueckii* also form lower levels of acetic acid than *S. cerevisiae.* No significant relationship between glycerol and acetic acid productions was observed in *T. delbrueckii* either (Renault et al. 2009). Therefore, it is possible that these yeasts display different metabolic responses to osmotic stress and redox imbalance. The production of other polyols (e.g. arabitol, mannitol and erythritol) in addition to glycerol has indeed been observed in various yeasts in response to osmotic stress (Tokuoka et al. 1992, van Eck et al. 1993, Kayingo et al. 2001). In the maintenance of redox balance, compounds such as higher alcohols, acetoin, 2,3-butanediol and organic acids can also be produced (Remize et al. 1999, Jain et al. 2012). Since the mentioned metabolites impact the wine quality and aroma, it is valuable to know which metabolites are produced in significant amounts.

In order to exploit the beneficial characteristics of the non-*Saccharomyces* yeasts, but to compromise for their low fermentation aptitudes, studies investigating the use of these yeasts in mixed cultures with *S. cerevisiae* strains have been conducted (Ciani et al. 2006, Romano et al. 2003). In mixed cultures, the yeast species do not co-exist passively, but rather interact with one another in various ways (Charoenchai et al. 1997, Hansen et al. 2001, Fleet 2003, Nissen and Arneborg 2003, Cheraiti et al. 2005). These interactions can have positive or negative effects on the yeasts and consequently on the wine. Furthermore, interactions on gene expression level have been reported (Milanovic et al. 2012, Sadoudi et al. 2014).

Since high acetic acid concentrations are detrimental to wine quality and other metabolites produced during wine fermentations might have an effect on the wine aroma, the metabolic response to the mentioned stresses in non-*Saccharomyces* yeasts require further research. Furthermore, the effect of mixed cultures on individual yeast metabolism and wine composition should be further investigated. Therefore, the objectives for this study were to (1) monitor the fermentation behaviour of the yeasts as pure or mixed starter cultures, (2) determine the concentrations of additional or alternative compatible solutes to glycerol and (3) investigate the production of glycerol and acetic acid on a molecular level in terms of the gene expression of *GPD1* and *ALD6.*

3.2 Materials and methods

3.2.1 Microorganisms used in this study

The yeast strains used in this work are shown in Table 1. The identities of *S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii* were confirmed by PCR-RFLP of the 5.8S rRNA-ITS region as described by Esteve-Zarzoso et al. (1999). Furthermore, in order to ensure that the *St. bacillaris* strain was not a *Starmerella bombicola* (formerly known as *Candida stellata*) strain,

the PCR-RFLP method proposed by Sipiczki (2004) was carried out. Propagation and maintenance of the yeast strains were performed in YPD (Biolab, Merck, Wadeville, South Africa). Agar (20 g/L) was added when necessary. The yeasts were stored as freeze-cultures at -80ºC in a 30% glycerol solution.

Escherichia coli DH5α *[F-j80lacZ∆ M15 ∆(lacZYA-argF) U169 deoR recA1 endA1 hsdR17(rk_, mk1) phoA supE44 thi-1 gyr96 relA1l]* was used for cloning and propagation of the selected genes. Luria Bertani (LB) broth (Biolab) and agar were used to culture the DH5α cells. Ampicillin (100 µg/mL; Sigma Aldrich, Johannesburg, South Africa) was added to the LB-agar plates for selection of the transformants.

Table 1 Yeast strains used during this study.

Strain[a]	Synonym/ Former name	Region/ Supplier
Saccharomyces cerevisiae **Lalvin EC1118**	-	Lallemand, Montreal, Canada.
Lachancea thermotolerans **IWBT Y1220**	Formerly: *Kluyveromyces thermotolerans*	Isolated from grape must in South Africa.
Torulaspora delbrueckii **CRBO L0544**	-	Isolated from grape must in France.
Starmerella bacillaris **IWBT Y1283**	Synonym: *C. zemplinina*	Isolated from grape must in South Africa.

[a]IWBT: Institute for Wine Biotechnology; CRBO: Centre de Ressources Biologiques Œnologie, Bordeaux, France

3.2.2 Fermentation conditions and sampling

Fermentations were performed with pure cultures of the yeast strains as well as mixed cultures of *S. cerevisiae* with each of the non-*Saccharomyces* yeasts (Table 2) in triplicate. Erlenmeyer flasks (with stoppers and fermentation caps) containing 350 mL synthetic grape must (modified from Henschke and Jiranek 1993; refer to Tables 3 and 4) were used as fermentation vessels. The medium was adjusted to a pH of 3.3 with potassium hydroxide (Saarchem, Krugersdorp, South Africa) and filter sterilized through 0.45-µm filters (Sartorius Stedim Biotech, Goettingen, Germany). The anaerobic factors were added after the medium was aliquoted into the fermentation flasks. The yeasts were inoculated (Table 2) into the medium after pre-culturing.The pre-culture strategy started with the inoculation of a colony of yeast culture into 5 mL YPD and incubated for 24 h at 30°C. Of these cultures, 2 mL were transferred into 200 mL YPD and incubated for 9 h at 30°C prior to inoculation.

In order to determine when the different cultures are in the exponential growth phase, growth curve experiments for the non-*Saccharomyces* yeasts were carried out. It was observed that all three yeasts are in exponential phase from approximately 4 to 10 h. Based on these results, it was decided to incubate the pre-cultures for 9 h at 30°C. The growth curve results were also used to determine the correlation between OD_{600nm} and cell concentration.

The fermentations were incubated at 25°C without agitation. Weight loss was monitored daily for the duration of the fermentations. A fermentation was considered complete when the 24-h weight difference was ≤0.1 g for two consecutive days.

Table 2 Summary of fermentation inoculation details.

Inoculation type	Species	Inoculation concentration
Pure cultures	*S. cerevisiae*	$2x10^6$ cells/mL
	L. thermotolerans	$2x10^6$ cells/mL
	T. delbrueckii	$2x10^6$ cells/mL
	St. bacillaris	$2x10^6$ cells/mL
Mixed cultures[a]	*S. cerevisiae* + *L. thermotolerans*	$1x10^6$ cells/mL of each
	S. cerevisiae + *T. delbrueckii*	$1x10^6$ cells/mL of each
	S. cerevisiae + *St. bacillaris*	$1x10^6$ cells/mL of each

[a] *S. cerevisiae* was inoculated 24 h later than the non-*Saccharomyces* yeasts.

Table 3 Chemical composition of the fermentation medium (modified from Henscke and Jiranek 1993).

	Amount per litre		Amount per litre
Carbon sources		**Vitamins**	
Glucose	115 g	Myo-inositol	100 mg
Fructose	115 g	Pyridoxine hydrochloride	2 mg
Acids		Nicotinic acid	2 mg
Potassium L-Tartrate	2.5 g	Calcium pantothenate	1 mg
L-Malic acid	3 g	Thiamin hydrocloride	0.5 mg
Citric acid	0.2 g	PABA.K	0.2 mg
Salts		Riboflavin	0.2 mg
Dipotassium phosphate	1.14 g	Biotin	0.125 mg
Magnesium sulphate heptahydrate	1.23 g	Folic acid	0.2 mg
Calsium chloride dihydrate	0.44 g	**Trace elements**	
Nitrogen sources		Manganese(II) chloride tetrahydrate	200 µg
Ammonium chloride	0.46 g	Zink chloride	135 µg
Amino acids	**(Table 3)**	Iron(II) chloride	30 µg
Anaerobic factors		Copper(II) chloride	15 µg
Ergosterol	10 mg	Boric acid	5 µg
Tween 80	0.5 mL	Cobalt(II) nitrate hexahydrate	30 µg
		Sodium molybdate dehydrate	25 µg
		Potassium iodate	10 µg

Samples (10 mL) were taken three times a week in duplicate from the fermentation flasks. The samples were centrifuged at 5000 rpm for 7 min. The supernatants were filtered through a 0.22-µm filter (Starlab Scientific, Cape Town, South Africa) and stored at -20°C before chemical analysis. The cell pellets were immediately frozen in liquid nitrogen and stored at -20°C before RNA extraction.

Table 4 Amino acid composition according to MS300 medium (Bely 1990).

Amino acid	mg/L	Amino acid	mg/L
Tyrosine	18.326	Alanine	145.299
Tryptophane	179.333	Valine	44.506
Isoleucine	32.725	Methionine	31.416
Aspartic acid	44.506	Phenylalanine	37.961
Glutamic acid	120.428	Serine	78.54
Arginine	374.374	Histidine	32.725
Leucine	48.433	Lysine	17.017
Threonine	75.922	Cystein	13.09
Glycine	18.326	Proline	612.612
Glutamine	505.274		

3.2.3 Enumeration of yeasts and analytical determinations

Yeast populations were monitored via the plate counting technique. WL agar (Fluka, Johannesburg, South Africa) was used for the enumeration of the pure cultures, the *S. cerevisiae-L. thermotolerans* and *S. cerevisiae-St. bacillaris* mixed cultures. YPD agar and YPD agar supplemented with 0.5 mg/L cycloheximide (Sigma Aldrich) were used to enumerate the *S. cerevisiae-T. delbrueckii* mixed cultures. *S. cerevisiae* counts were given as the difference between the counts on the YPD plates and the cycloheximide supplemented plates.

Aliquots of the filtered supernatants throughout fermentation were sent to the Central Analytical Facility (Stellenbosch University, Stellenbosch, South Africa) for the determination of residual glucose and fructose, glycerol and acetic acid concentrations. The Arena 20XT Photometric Analyzer (Thermo Electron Oy, Finland) and enzyme kits from Thermo Fisher Scientific (Johannesburg, South Africa) were used for the analysis.

Polyol (mannitol+arabitol and sorbitol+xylitol) concentrations from days 5, 12 and the end of fermentation were determined enzymatically with kits from Megazyme (Wicklow, Ireland) and used according to the manufacturer's instructions. The individual polyols (mannitol and arabitol; sorbitol and xylitol) could not be distinguished by the enzyme kits, therefore the results were given as a mixture of the two polyols.

For the analysis of the volatile compounds (Table 5) from end of fermentation samples, gas chromatography coupled with flame ionisation detection (GC-FID) was used. The extraction and operating methods were carried out according to Louw et al. (2009) with minor modifications. After the sample/ether mixture was centrifuged at 4000 rpm for 3 min, sodium sulphate was added and the centrifugation step repeated. The extracted samples were injected in duplicate.

Ethanol and succinic acid concentrations of end-point samples were determined through HPLC. Sample and standard preparation, method and instrument details were carried out and

set according to Eyéghé-Bickong et al. (2012), with a minor modification: the addition of ethanol to the calibration standards (20 g/L). The ethanol concentrations in the mixed culture fermentations were also measured at the time where *S. cerevisiae* starts to dominate or the non-*Saccharomyces* cell counts start to decline (day 9).

Table 2 Volatile compounds determined by GC-FID.

Higher alcohols	Ethyl esters	Acetate esters	Volatile fatty acids	Carbonyl compound
2-Phenylethanol	Ethyl hexanoate	Ethyl acetate	Iso-butyric acid	Acetoin
Hexanol	Diethyl succinate	Hexyl acetate	Butyric acid	
Methanol	Ethyl caprate	Isoamyl acetate	Iso-valeric acid	
Propanol	Ethyl caprylate	2-Phenylethyl acetate	Valeric acid	
Isobutanol	Ethyl lactate		Hexanoic acid	
Butanol	Ethyl butyrate		Octanoic acid	
Isoamyl alcohol	Ethyl phenylacetate		Decanoic acid	
Pentanol	Ethyl-3-hydroxybutanoate		Propionic acid	
3-Ethoxy-1-propanol				

3.2.4 Statistical analysis

The fermentation metabolites measured for all the fermentation cultures were compared to those of *S. cerevisiae* pure culture fermentation through t-tests in a custom-built perl program for each fermentation culture. The comparisons were visualized as networks with Cytoscape (version 2.8.2, available at www.cytoscape.org). The network graphs only include information that was found to be significantly different ($p<0.05$) from *S. cerevisiae*.

Furthermore, Principal component analysis (PCA) was performed using The Unscrambler (version 9.2, CAMO ASA, Norway) to illustrate the distribution of the fermentation cultures based on the residual sugar concentrations and metabolite production.

3.2.5 Amplification, cloning and sequencing of selected genes

Genomic DNA was extracted from *S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii* as described in Short protocols in Molecular Biology (Ansubel et al. 2002). The extracted DNA was resuspended in 40 µL milli-Q water (Merck Millipore, Billerica, USA). Selected genes (*GPD1, GPD2, GPP1, GPP2,* and *ALD6*) were amplified from the extracted genomic DNA by PCR (Refer to Table 6 for primer sequences). Phusion High-Fidelity DNA polymerase (Thermo Scientific) was used in the PCR for its proofreading ability (Refer to Table 7 for PCR programs) according to the manufacturer's instructions. The PCR products were visualized on a 1% agarose (SeaKem LE Agarose, Lonza) gel containing ethidium bromide, with the O'GeneRuler 1 Kb (Fermentas, Pretoria, South Africa) molecular marker.

The *GPD1, GPD2, GPP1, GPP2* and *ALD6* genes of each yeast strain were cloned into pJET cloning vector using the CloneJET PCR Cloning kit (Thermo Scientific). The recombinant plasmids were transformed into *E. coli* DH5α chemically competent cells.

Table 6 Primer sequences for the amplification of the selected genes.

PCR program nr[b]	Name	Sequence (5'-3')	Amplicon size (bp)	Purpose[a]
1	Gpd1Scfw	ATGTCTGCTGCTGCTGATAGATT	1176	Amplification of *GPD1* in Sc
	Gpd1Scrv	CTAATCTTCATGTAGATCTAATTCTTC		
	Gpd1Tdfw	ATGGCCGCTTCTGACAGACT	1188	Amplification of *GPD1* in Td
	Gpd1Tdrv	TCAATGTGCGGACTCCTTCAAT		
5	Gpd1Ltfw	ATGTTTTCAATCTCCAGAATCACTAG	1293	Amplification of *GPD1* in Lt
	Gpd1Ltrv	TTATTCGCTGTGCTCGCCTTC		
8	Gpd2Scfw	ATGCTTGCTGTCAGAAGATTAACAA	1323	Amplification of *GPD2* in Sc
	Gpd2Scrv	CTATTCGTCATCGATGTCTAGCT		
2	Gpp1Scfw	ATGCCTTTGACCACAAAACCTTT	753	Amplification of *GPP1* in Sc
	Gpp1Scrv	TTACCATTTCAACAAGTCATCCTTA		
3	Gpp1Tdfw	ATGCCATTGACTGCCAAACCA	750	Amplification of *GPP1* in Td
	Gpp1Tdrv	TTACCATTTCAACAAATCGTCTTTG		
6	Gpp1Ltfw	ATGTCTTCCTCCAAGCCCATC	750	Amplification of *GPP1* in Lt
	Gpp1Ltrv	TTACCACGTCAAGACGTCGTC		
7	Gpp2Scfw	ATGGGATTGACTACTAAACCTCTA	753	Amplification of *GPP2* in Sc
	Gpp2Scrv	TTACCTTTCAACAGATCGTCCTTA		
	Gpp2Tdfw	ATGCCTTTGACTACCAAACCA	753	Amplification of *GPP2* in Td
	Gpp2Tdrv	TTACCATTTTAGCAAGTCGTCCTT		
	Gpp2Ltfw	ATGCCTCTATCCAAGCCTCTA	750	Amplification of *GPP2* in Lt
	Gpp2Ltrv	TTACCATTGCAACAAGTCGTCC		
4	Ald6Scfw	ATGACTAAGCTACACTTTGACACT	1047	Amplification of *ALD6* in Sc
	Ald6Scrv	TTACAACTTAATTCTGACAGCTTTTAC		
1	Ald6Ltfw	ATGAATTACGCCTGTCTAAGAAG	1047	Amplification of *ALD6* in Lt
	Ald6Ltrv	CTAAGCAAGCTTGATTCTAACTG		
4	Ald6Tdfw	ATGGCTCAATACAAGACTACAGC	1056	Amplification of *ALD6* in Td
	Ald6Tdrv	AATTTAATTCTGATGGCCTTCACG		
	Ald6ScInt	GGTGACGGCTACATGAACTTCA	-	Sequencing an internal part of *ALD6* of Sc
	Ald6TdInt	CCACTCTGGTGACACTCACAT	-	Sequencing an internal part of *ALD6* of Td

[a]Sc: *Saccharomyces cerevisiae*; Lt: *Lachancea thermotolerans*; Td: *Torulaspora delbrueckii*

[b]: Refer to Table 7

The competent cells were prepared as follows: cells were grown overnight in 5 mL 2x LB medium containing 0.2% glucose at 30°C. The cells were then transferred into 100 mL 2x LB containing 0.2% glucose and incubated at 30°C on a rotary shaker until an optical density (OD_{600nm}) of 0.3-0.5 was reached. Subsequently, the cells were placed on ice for 2 h and

transferred to two sterile falcon tubes (50 mL each). The tubes were centrifuged at 3000 g for 5 min at 4°C. The supernatant was discarded and the cells resuspended in 25 mL competency buffer (0.1 M $CaCl_2$, 0.07 M $MnCl_2$, 0.04 M NaOAc) followed by an incubation step of 30 min on ice. Again the cells were centrifuged at 3000 g for 5 min at 4°C and the supernatant discarded. Finally the cells were resuspended in 2.5 mL of the competency buffer and the content of the two tubes added together after which 1.15 mL 80% glycerol was added. Competent cells were stored at -80°C until further use was required. The transformation was performed according to the heat shock method. 10 µL of the ligation reaction was added to 50 µL of competent cell suspensions and incubated for 45 min on ice after which it was heat shocked for 2 min at 42°C in a water bath. The heat shocked cells were immediately placed on ice for 5 min. Subsequently, 950 µL LB medium was added and the cells were regenerated at 37°C for 1.5 h with shaking. The regenerated cells were plated out on LB agar plates supplemented with ampicillin and incubated overnight at 37°C.

Table 7 PCR programs for the selected genes.

Nr	Genes[b]	PCR thermocycling conditions				
		Initial denaturation	30 cycles			Final elongation
			Denaturation	Annealing	Elongation	
1	*ScGPD1*	98ºC;30s	98ºC;10s	57ºC;30s	72ºC;40s	72ºC;10min
	TdGPD1					
	LtALD6					
2	*ScGPP1*			55ºC;30s	72ºC;25s	
3	*TdGPP1*			57ºC;30s	72ºC;30s	
4	*ScALD6*			57ºC;30s	72ºC;60s	
	TdALD6					
5	*LtGPD1*[a]			60ºC;30s	72ºC;40s	
6	*LtGPP1*[a]			60ºC;30s	72ºC;25s	
7	*TdGPP2*			56ºC;30s	72ºC;30s	
	LtGPP2[a]					
	ScGPP2					
8	*ScGPD2*			52ºC;30s	72ºC;30s	

[a]Each of these genes showed two bands on the gel after PCR run, so the correct band was excised for further isolation.
[b]Sc: *Saccharomyces cerevisiae*; Lt: *Lachancea thermotolerans*; Td: *Torulaspora delbrueckii.*

In order to verify the transformation, plasmid DNA extractions and restriction digests were performed. The plasmid DNA extraction was performed using the PureYield Plasmid Miniprep Kit (Promega, Johannesburg, South Africa) and the plasmids were digested with *Bgl*II (Roche, Johannesburg, South Africa) according to the manufacturer's instructions in order to release the inserted gene. The cloning vector with the gene insert was sent for sequencing at

the Central Analytical Facility (Stellenbosch University). The gene sequence identities were confirmed by using the BLAST tool (available at http://blast.ncbi.nlm.nih.gov/) and the known genomes in the NCBI database. The amino acid sequences were also obtained by translating the gene sequences in silico using the ExPASy Translate tool (available at web.expasy.org/translate/). Thereafter, the nucleotide and amino acid sequences were aligned between the three strains using the ClustalW software (available at http://www.genome.jp/tools/clustalw/).

3.2.6 RNA isolation and cDNA synthesis

The total RNA of all the samples were extracted for days 2, 7 and 12 of the fermentation using a hot phenol method with glass beads adapted from Current protocols in molecular biology (Collart and Oliviero 1993). The frozen cell pellets were defrosted on ice and washed with 1 mL milliQ water. The pellet was resuspended in 400 µL milli-Q water and transferred to screw cap tubes with 200 µL acid washed glass beads (Merck, Modderfontein, South Africa). The tubes were vortexed for 2 min 30 s after which 400 µL liquefied phenol (Sigma Aldrich) was added. The tubes were inverted vigorously to mix the contents. The samples were incubated for 30 min at 65°C in a water bath and vigorously inverted every 10 min. A 5-min incubation step on ice followed, after which the samples were centrifuged for 5 min at 4°C (15000 rpm). The aqueous phase was transferred into a new eppendorf tube, 400 µL phenol was added and samples inverted vigorously to mix the contents. The samples were incubated on ice again and centrifuged as mentioned above. The aqueous layer was transferred to a new tube and 400 µL chloroform (Merck) was added. The samples were mixed, incubated on ice, centrifuged and the aqueous layer transferred to a new tube as before. For the precipitation, 40 µL of 3 M sodium acetate (Sigma Aldrich) was added together with 1 mL absolute ethanol (Sigma Aldrich) subsequently the samples were gently inverted to mix. A centrifugation step followed (5 min, 15000 rpm, 4°C), after which the supernatants were discarded and the pellets washed with 70 % ethanol. Subsequently, they were centrifuged as mentioned above and the supernatants discarded. The pellets were dried in a fume hood for approximately 10-15 min and resuspended in 15 µL milli-Q water. 1 µL RiboLock RNase Inhibitor (40 U/µL; Thermo Scientific) was added.

RNA concentrations and purity were determined by using the NanoDrop ND-1000 (Thermo Fisher Scientific). The RNA samples were treated with DNaseI recombinant enzyme (Roche) according to the manufacturer's instructions to eliminate the presence of DNA.

Reverse transcription was performed using the ImProm-II Reverse Transcription System (Promega) with the Oligo$(dT)_{15}$ primer supplied with the kit according to the manufacturer's instructions.

3.2.7 Primer design and RT-qPCR

Species specific primers were designed (Table 8) in variable regions of the genes (determined by multiple alignment analysis). The Primer3 software (available at http://simgene.com/Primer3) was applied to design primers of 20 bp with a melting temperature of 60ºC.

Real time PCR was carried out on the Applied Biosystems 7500 Real Time PCR system using KAPA SYBR FAST Universal qPCR Kit (KAPA Biosystems, Cape Town, South Africa). The reactions were set up in 96-well reaction plates (Applied Biosystems). The program used was the following: 95°C for 3 min; 40 cycles of 95°C for 15 s, 60°C for 1 min. A dissociation cycle was added after the amplification. For calibration using LRE-based methods (Rutledge and Stewart 2010), Lambda genomic DNA (250 ng/µL; Roche) was used. The DNA was diluted to 100 fg/µL. Primers amplifying a 151-bp fragment of the Lambda DNA known as the CAL1 amplicon was used.

Table 8 RT-qPCR primer sets

Name	Sequence (5'-3')	Amplicon length (bp)	Purpose[a]
ScGPD1qPCRfw	GGTTGGAAACATGTGGCTCT	91	Amplification of *GPD1* in Sc
ScGPD1qPCRrv	GGCAGGTTCTTCATTGGGTA		
LtGPD1qPCRfw	CACTTGTTCGTCAAGCGTGT	89	Amplification of *GPD1* in Lt
LtGPD1qPCRrv	TGGTGCTTGGTGTTGATGAT		
TdGPD1qPCRfw	AGATTCCGTCAAGGATGTGG	135	Amplification of *GPD1* in Td
TdGPD1qPCRrv	CCAACTTCGAAACCCTTCAA		
ScGPD2qPCRfw	CCCAGAATCCAAAGTCGAAA	131	Amplification of *GPD2* in Sc
ScGPD2qPCRrv	CTTCCAAGGCTGACTTACCG		
ScALD6qPCR2fw	CCTTCCACTGAAAACACCGT	108	Amplification of *ALD6* in Sc
ScALD6qPCR2rv	GGTAGCCCATTCAGTGTCGT		
LtALD6qPCR2fw	AGCTCGCTCACTTCTCAAGC	168	Amplification of *ALD6* in Lt
LtALD6qPCRrv	CCATCAAGCTTGTCTGCGTA		
TdALD6qPCRfw	CATTGAGCCCACCATCTTTT	124	Amplification of *ALD6* in Td
TdALD6qPCRrv	ATTTGCCAACTCGACAGCTT		
F1	AGACGAATGCCAGGTCATCTGAAACAG	151	Amplification of the CAL amplicon in Lambda
R1	CTTTTGCTCTGCGATGCTGATACCG		

[a]Sc: *Saccharomyces cerevisiae*; Lt: *Lachancea thermotolerans*; Td: *Torulaspora delbrueckii*

The PCR efficiencies of the primer pairs and the specificity were determined by amplifying serial dilutions of genomic DNA of the yeast strains. The primers were tested against all three strains to ensure species specificity. PCR efficiency was calculated through the equation $E = -1+10^{[-1/slope]}$.

3.3 Results

3.3.1 Confirmation of species identity

PCR-RFLP was applied to confirm the identities of the yeast species used in the study. The ITS-region of the 5.8S rRNA was amplified and the PCR products were digested. For *S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii* the enzymes *Hae*III, *Cfo*I and *Hinf*I were used. Digestion with these enzymes was reported to lead to unique restriction patterns for identification of these yeast species (Esteve-Zarzoso et al. 1999). The sizes of the PCR products and the banding patterns of the restriction digests were compared to a study conducted by Esteve-Zarzoso et al. (1999) and the identities of the yeasts were thus confirmed. In the case of *St. bacillaris*, the PCR-RFLP method as described by Sipiczki et al. (2004) was used to ensure that this yeast is indeed *St. bacillaris* and not *St bombicola.*

3.3.2 Fermentation results

3.3.2.1 Fermentation kinetics and population dynamics

The *S. cerevisiae* pure culture fermentation, which also served as control, completed alcoholic fermentation i.e. fermented to dryness within 16 days (Table 9, Fig. 1). By contrast, none of the non-*Sacharomyces* yeasts was able to complete fermentation in the conditions used here; the fermentations were stuck. Fermentations were considered stuck when less than 0.1 g weight difference between consecutive days was observed.

The fermentations inoculated with *L. thermotolerans*, *T. delbrueckii* and *St. bacillaris* reached residual sugar concentrations of 70.77 g/L, 85.69 g/L and 107.39 g/L, respectively (Table 9). The sugar consumption pattern (Fig. 1) indicated that *L. thermotolerans* and *T. delbrueckii,* like *S. cerevisiae*, utilized glucose preferentially to fructose, although at a slower rate. In contrast, *St. bacillaris* consumed fructose preferentially to glucose. It is indeed known that this yeast is fructophilic (Sipizcki 2004). However, the fructophilic character of this strain of *St. bacillaris* was particularly acute as it only consumed about 10 g/L glucose (Fig. 1).

L. thermotolerans had the lowest cell count of all the pure cultures and started to die off earlier than the others (Fig. 2A). Although *T. delbrueckii* reached the highest cell numbers, this yeast did not persist long at that cell concentration; the cell count started to decline after day 9 of the fermentation (Fig. 2A). The *St. bacillaris* pure culture fermentation reached cell numbers similar to that of *S. cerevisiae* and did not start to decrease before the fermentation was stopped (Fig. 2A).

The mixed culture fermentations reached dryness a few days after the control (Fig. 1). The sugars were consumed at a slower rate than *S. cerevisiae* while the non-*Saccharomyces* were present in high concentrations (Fig. 1). However, since the non-*Saccharomyces* yeasts did

not persist until the end of fermentation in high cell concentrations (Fig. 2B-D), it can be assumed that it was *S. cerevisiae* that completed the fermentation. *L. thermotolerans* could only be detected until day 12 of the fermentation (Fig. 2B). Furthermore, *L. thermotolerans* cell counts declined even before *S. cerevisiae* started to dominate. *T. delbrueckii* and *St. bacillaris* persisted longer in the fermentation with *S. cerevisiae*, but the cell counts declined as soon as *S. cerevisiae* started to dominate (Fig. 2C, D). Furthermore, the *S. cerevisiae* cell count did not reach the same level in the mixed culture as in the pure culture fermentation (Fig. 2B-D).

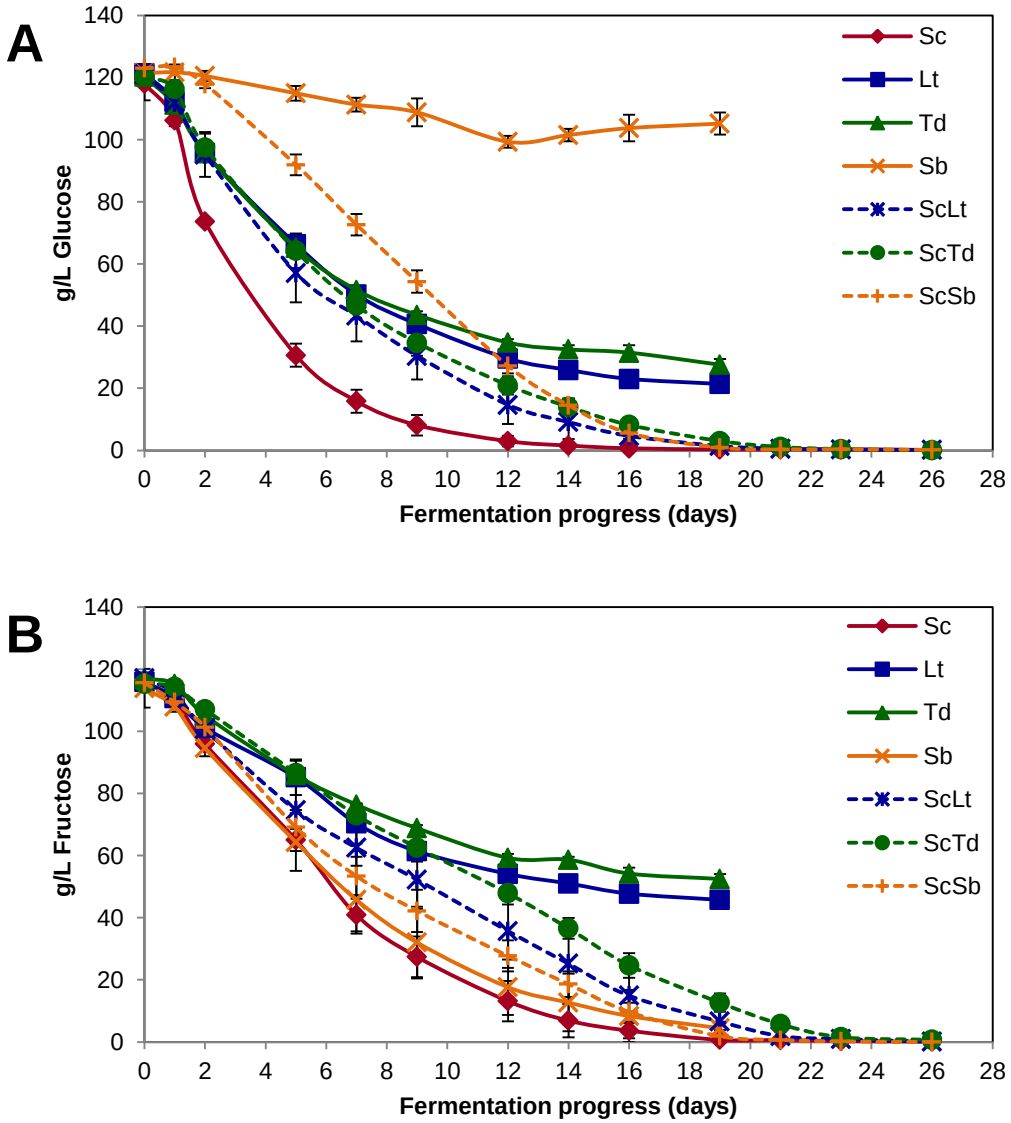

Fig. 1 Sugar consumption throughout fermentation. A: Glucose consumption; B: Fructose consumption. Sc: *S. cerevisiae*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sb: *St. bacillaris*.

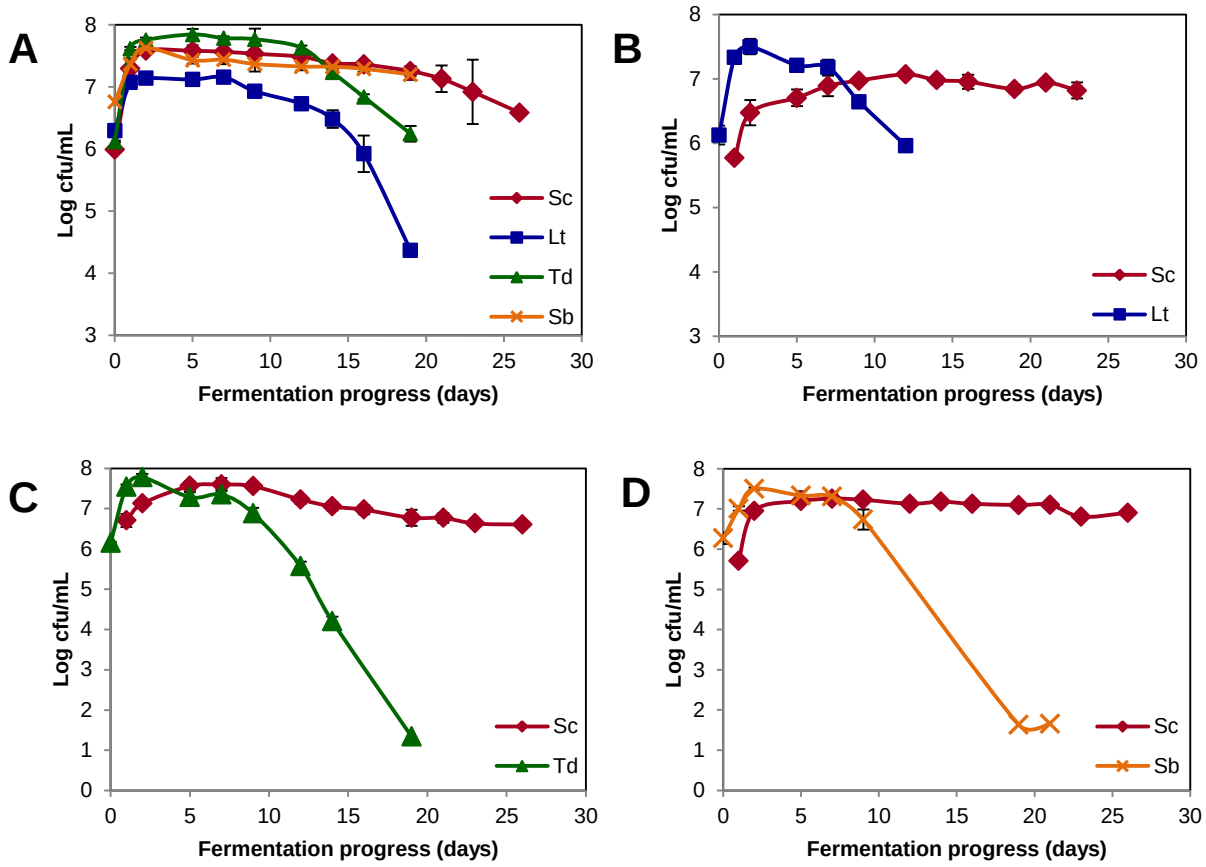

Fig. 2 Cell counts throughout fermentation. A: Pure culture fermentations; B: *S. cerevisiae-L. thermotolerans* mixed culture; C: *S. cerevisiae-T. delbrueckii* mixed culture; D: *S. cerevisiae-St. bacillaris* mixed culture. Sc: *S. cerevisiae*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sb: *St. bacillaris*.

Table 9 End of fermentation concentrations of primary fermentation products (g/L except ethanol).

	Sc	Lt	Td	Sb	ScLt	ScTd	ScSb
Residual sugars[a]	0.23	70.77	85.69	107.39	0.29	0.86	0.19
Glucose	0.11±0.04	23.02±1.19	31.48±2.37	103.73±4.27	0.17±0.03	0.14±0.03	0.17±0.03
Fructose	0.12±0.06	47.75±1.17	54.22±1.86	3.66±2.69	0.11±0.03	0.71±0.13	0.02±0.01
Ethanol[b]	15.26±0.07	10.62±0.24	9.80±0.17	8.09±0.50	14.34±0.19	13.82±0.76	14.92±0.11
Polyols							
Glycerol	6.51±0.05	5.13±0.07	6.48±0.21	9.17±0.42	7.19±0.26	7.74±0.16	8.51±0.20
Mannitol + Arabitol[c]	0.08±0.002	0.11±0.009	0.11±0.03	0.02±0.007	0.25±0.01	0.29±0.02	0.04±0.0005
Sorbitol + Xylitol[d]	0.03±0.009	0.12±0.01	0.15±0.02	0.09±0.01	0.10±0.04	0.14±0.02	0.06±0.005
Acids							
Acetic acid	0.90±0.02	0.22±0.03	0.62±0.002	0.55±0.01	0.64±0.02	0.82±0.03	0.84±0.03
Succinic acid	0.40±0.006	0.39±0.01	0.61±0.03	0.19±0.01	0.50±0.01	0.63±0.06	0.42±0.02

[a] Glucose + Fructose; [b] %v/v; [c] Expressed as g/L mannitol; [d] Expressed as g/L sorbitol.
Sc: *S. cerevisiae*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sb: *St. bacillaris*

3.3.2.2 Primary fermentation metabolites (including certain polyols)

The concentrations of the primary metabolites produced in all the fermentations are presented in Table 9 and Figures 3-5, and their yields per gram of sugar consumed are shown in Table 10.

The ethanol concentrations of the non-*Saccharomyces* pure culture fermentations were considerably lower than that of *S. cerevisiae* (Table 9, Fig. 3), but the production yields were similar (Table 10). Therefore, the low ethanol level can be attributed to the low sugar consumption, especially for *St. bacillaris.*

Table 10 Yields of primary fermentation metabolites in mg/g sugars consumed

	Sc	Lt	Td	Sb	ScLt	ScTd	ScSb
Ethanol	515.46±14.79	503.53±9.58	510.22±9.18	520.45±53.26	475.74±6.89	464.39±25.29	493.62±0.56
Polyols							
Glycerol	27.86±0.49	30.80±0.23	42.81±2.16	74.81±7.46	30.25±1.02	32.97±0.69	35.70±1.04
Mannitol + Arabitol	0.35±0.02	0.63±0.05	0.71±0.16	0.18±0.04	1.04±0.06	1.22±0.10	0.17±0.001
Sorbitol + Xylitol	0.12±0.04	0.72±0.07	1.01±0.11	0.78±0.17	0.44±0.15	0.61±0.07	0.26±0.02
Acetic acid	3.83±0.2	1.34±0.15	4.07±0.1	4.46±0.31	2.69±0.09	3.51±0.14	3.53±0.16

Sc: *S. cerevisiae*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sb: *St. bacillaris*

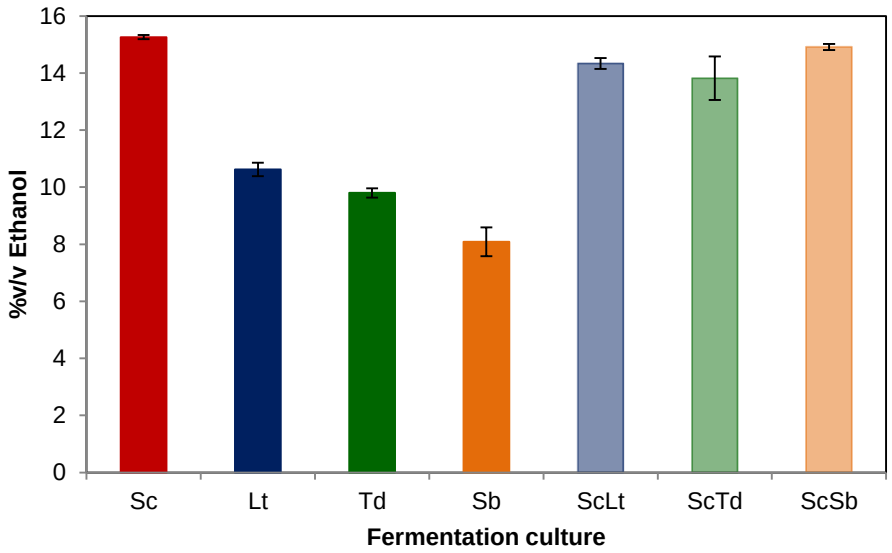

Fig. 3 Ethanol concentrations at the end of fermentation. Sc: *S. cerevisiae*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sb: *St. bacillaris*.

Regarding glycerol concentration, *S. cerevisiae* produced most of the glycerol at the beginning of fermentation, while the non-*Saccharomyces* yeasts produced it more gradually (Fig. 4). *L. thermotolerans* produced less glycerol than *S. cerevisiae*, but the production yields

were similar. *T. delbrueckii* pure culture fermentation produced similar amounts of glycerol than the control fermentation (Fig. 4, Table 9). However, *T. delbrueckii* produced more glycerol per amount of sugar consumed (Table 10). Even with the low sugar consumption compared to the other fermentations, *St. bacillaris* produced significant amounts of glycerol (Table 9, Fig. 4). Concerning the other polyols measured, *L. thermotolerans* produced higher total amounts and higher yields than *S. cerevisiae* (Table 9, 10). Furthermore, *T. delbrueckii* produced more of the other polyols measured than *S. cerevisiae,* especially in the case of sorbitol+xylitol (Table 9). Moreover, the production yields were considerably higher (Table 10). In contrast to the other two non-*Saccharomyces* species, *St. bacillaris* did not produce more mannitol+arabitol than the control (Table 9). Concerning sorbitol+xylitol, it did produce slightly more than *S. cerevisiae* (Table 9).

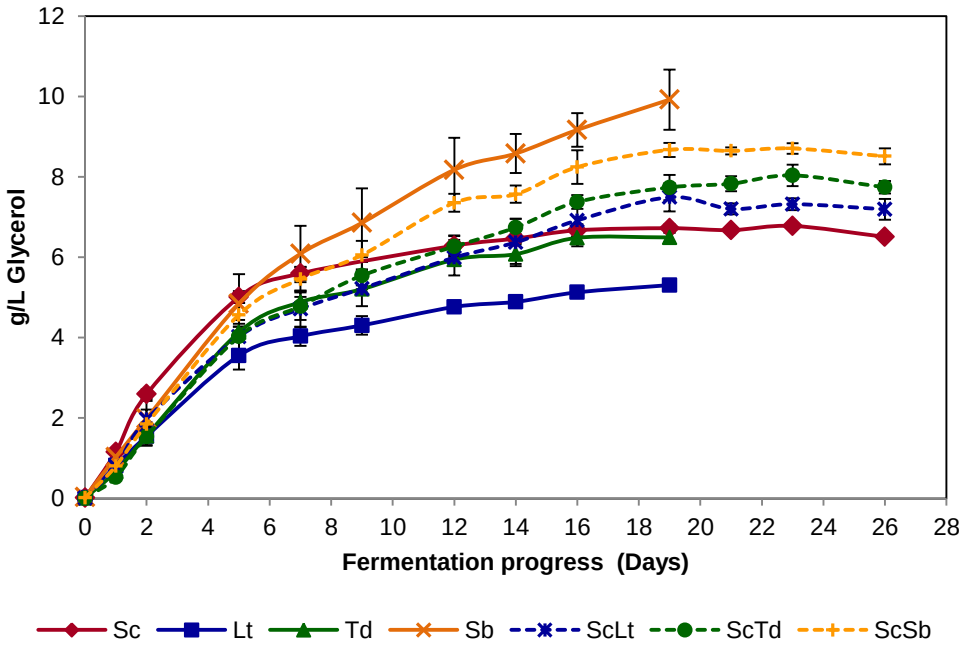

Fig. 4 Glycerol concentrations throughout fermentation. Sc: *S. cerevisiae*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sb: *St. bacillaris*.

St. bacillaris produced the lowest and *T. delbrueckii* the highest amounts of succinic acid, while *L. thermotolerans* produced similar amounts as the control (Table 9). The production of acetic acid in the non-*Saccharomyces* pure cultures was gradual throughout the fermentation, unlike *S. cerevisiae* that produced most in the first few days (Fig. 5). The final acetic acid concentrations in the *T. delbrueckii* and *St. bacillaris* pure culture fermentations were lower than *S. cerevisiae*, yet the yields were higher, thus these yeasts have the potential to produce high levels of acetic acid. In contrast, the *L. thermotolerans* fermentation resulted in noticeably lower acetic acid concentrations (Table 9, Fig. 5) as well as yield (Table 10). At approximately day 12, the cell counts of *T. delbrueckii* suddenly started to decline, and at that

point, the acetic acid concentration in this fermentation increased (Fig. 2A, Fig. 5), while in the other fermentations the acetic acid levels started to plateau.

In the fermentations where *L. thermotolerans* and *St. bacillaris* were respectively inoculated together with *S. cerevisiae*, the final ethanol concentrations were similar to that of the control (Table 9). The mixed culture with *T. delbrueckii* resulted in a lower ethanol concentration than the *S. cerevisiae* pure culture fermentation. It can be assumed that most of the ethanol produced at the end of the mixed culture fermentations was an outcome of *S. cerevisiae*'s metabolism, because the cell concentrations of the non-*Saccharomyces* yeasts started to decrease early in the fermentation (Fig. 2B-D).

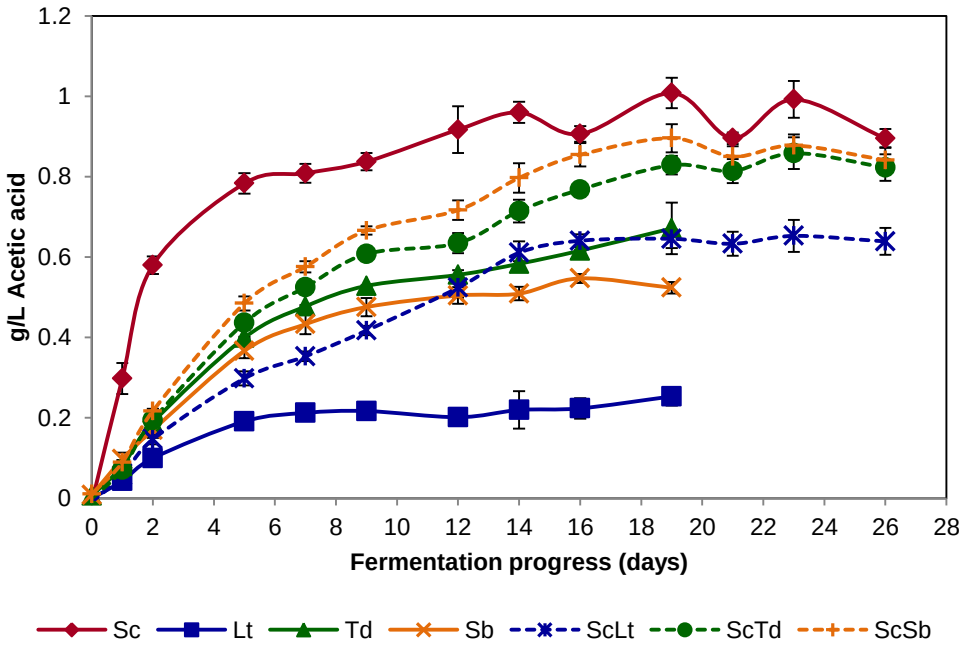

Fig. 5 Acetic acid concentrations throughout fermentation.
Sc: *S. cerevisiae*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sb: *St. bacillaris*.

All the mixed culture fermentations resulted in higher glycerol concentrations in comparison to the control. Furthermore, the mixed fermentation with *St. bacillaris* produced the highest concentration of glycerol; it is not surprising since *St. bacillaris* produced high levels on its own. The production rate followed the trend of pure *St. bacillaris* until day 5 (Fig. 4). This corresponds approximately to the time when *S. cerevisiae* reached the same cell count in the mixed culture as *St. bacillaris* (Fig. 2D). Consequently, the production of glycerol was lower than what was observed for the pure culture of *St. bacillaris* (Fig. 4). In the case of the mixed culture fermentations with *L. thermotolerans* and *T. delbrueckii,* the levels were higher than in either one of the pure cultures (Fig. 4). Furthermore, the mixed culture fermentations followed the production trend of these non-*Saccharomyces* yeasts initially, before *S. cerevisiae* started to dominate.

Table 11 Concentrations of major volatile compounds at end of fermentation (mg/L).

	Sc	Lt	Td	Sb	ScLt	ScTd	ScSb
Higher alcohols	153.81	147.07	99.33	86.15	286.18	280.22	205.60
Isobutanol	16.69±0.25	9.58±1.41	12.24±0.41	26.31±5.53	24.57±0.42	28.03±1.48	24.19±0.01
Propanol	31.27±3.65	44.64±2.6	13.90±0.36	22.03±0.31	81.66±7.78	67.56±1.85	44.87±0.79
2-Phenyl ethanol	19.54±0.03	21.69±1.81	12.08±0.75	10.03±1.37	27.87±1.23	30.54±2.44	24.89±0.32
Isoamyl alcohol	74.76±3.19	62.93±4.69	50.16±1.68	27.78±3.69	142.69±2.64	138.78±17.54	104.41±6.07
Butanol	0.60±0.01	8.23±1.69	0.70±0.01	nd[a]	2.84±0.76	1.16±0.08	0.66±0.02
Hexanol	0.65±0.02	nd	nd	nd	0.70±0.03	0.70±0.03	0.67±0.0005
3-Ethoxy-1-propanol	10.30±0.34	nd	10.25±0.28	nd	5.85±0.37	13.45±2.88	5.91±0.41
3-Methyl-1-pentanol	nd	nd	nd	nd	nd	nd	nd
Methanol	nd	nd	nd	nd	nd	nd	nd
Pentanol	nd	nd	nd	nd	nd	nd	nd
Acetate esters	34.87	45.76	39.77	19.96	56.11	58.88	58.00
Ethyl acetate	32.89±2.74	44.68±3.29	39.10±0.74	19.33±1.39	54.76±9.59	57.48±1.26	56.22±1.43
2-Phenylethyl acetate	1.64±0.03	0.76±0.02	0.67±0.002	0.63±0.003	0.93±0.003	1.00±0.05	1.39±0.02
Isoamyl acetate	0.34±0.0006	0.32±0.01	nd	nd	0.42±0.03	0.40±0.02	0.39±0.01
Hexyl acetate	nd	nd	nd	nd	nd	nd	nd
Ethyl esters	13.63	12.75	1.56	11.5	13.76	13.72	13.61
Ethyl phenylacetate	2.37±0.04	2.17±0.02	1.47±0.02	1.17±0.01	2.50±0.16	2.01±0.14	2.03±0.008
Ethyl lactate	10.39±0.001	10.42±0.03	nd	10.33±0.002	10.63±0.02	11.11±0.19	10.84±0.03
Ethyl caprate	0.23±0.03	0.16±0.03	0.09±0.003	nd	0.14±0.04	0.10±0.006	0.10±0.004
Ethyl hexanoate	0.53±0.0008	nd	nd	nd	0.49±0.004	0.50±0.006	0.55±0.002
Ethyl caprylate	0.11±0.003	nd	nd	nd	nd	nd	0.09
Ethyl butyrate	nd	nd	nd	nd	nd	nd	nd
Ethyl-3-hydroxybutanoate	nd	nd	nd	nd	nd	nd	nd
Diethyl succinate	nd	nd	nd	nd	nd	nd	nd
Short chain fatty acids	5.23	3.91	5.42	5.36	6.60	8.42	6.96
Isobutyric acid	1.23±0.0006	0.92±0.07	2.18±0.06	2.33±0.40	1.38±0.13	2.68±0.21	1.80±0.10
Butyric acid	0.94±0.01	0.61±0.004	0.66±0.006	0.65±0.01	0.76±0.02	0.93±0.05	1.03±0.009
Propionic acid	1.75±0.06	1.47±0.07	1.51±0.05	1.34±0.07	3.04±0.16	3.15±0.23	2.33±0.07
Isovaleric acid	1.31±0.02	0.91±0.01	1.07±0.03	1.04±0.02	1.42±0.04	1.66±0.05	1.80±0.02
Valeric acid	nd	nd	nd	nd	nd	nd	nd
Medium chain fatty acids	6.20	3.93	2.97	2.12	4.66	3.96	4.81
Octanoic acid	2.57±0.15	1.17±0.04	1.10±0.006	1.00±0.007	1.44±0.008	1.65±0.12	2.22±0.01
Decanoic acid	2.46±0.25	2.10±0.28	1.18±0.01	1.12±0.009	2.51±0.47	1.58±0.15	1.87±0.06
Hexanoic acid	1.17±0.08	0.66±0.004	0.69±0.006	nd	0.71±0.02	0.73±0.02	0.72±0 004
Carbonyl compound							
Acetoin	10.82±0.26	20.64±2.35	7.45±0.44	94.24±4.29	7.90±1.63	10.92±1.82	8.16±0.99

[a]nd: not detected; Sc*: S. cerevisiae*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sb: *St. bacillaris*

Concerning the other polyols measured the mixed culture fermentations with *L. thermotolerans* and *T. delbrueckii* produced more polyols than *S. cerevisiae*. The mannitol+arabitol concentration was higher than either the pure cultures of the yeasts involved. The sorbitol+xylitol concentration was lower in the *S. cerevisiae-L. thermotolerans* mixed culture than in the *L. thermotolerans* pure culture. The sorbitol+xylitol concentration was similar to the *T. delbrueckii* pure culture. Given that *S. cerevisiae* as well as *St. bacillaris* pure culture fermentations produced little of these polyols the mixed culture also produced low levels (Table 9). The mixed culture fermentation with *St. bacillaris* resulted in lower mannitol+arabitiol and higher sorbitol+xylitol concentrations than the control. The opposite was observed when compared to the pure *St. bacillaris* fermentation.

The final acetic acid concentrations of the mixed cultures with *T. delbrueckii* and *St. bacillaris* respectively, were similar to that of the *S. cerevisiae* pure culture (Table 9). Furthermore, the acetic acid production trend followed that of the non-*Saccharomyces* until *S. cerevisiae* started to dominate and the non-*Saccharomyces* cell counts decreased (Fig. 5, Fig. 2C, D). The acetic acid concentration in the mixed culture with *L. thermotolerans* was significantly lower than that of the control. It is worthwhile to note that while *L. thermotolerans* was present in the fermentation, the acetic acid levels increased slowly but rose more rapidly when *L. thermotolerans* started to die off and *S. cerevisiae* took over. Therefore, the presence of *L. thermotolerans* lowered the acetic acid produced by *S. cerevisiae*, especially in the beginning, because *S. cerevisiae* produced most of its acetic acid during the first five days, while the other cultures produced it more gradually (Fig. 5).

3.3.2.3 Volatile metabolites

The final concentrations of volatile aroma compounds are presented in Table 11 and Figures 6 and 7.

The total higher alcohol concentrations of the non-*Saccharomyces* pure cultures were less than that of the control (Table 11). However, the propanol and butanol concentrations of *L. thermotolerans* were higher than *S. cerevisiae* (Table 11, Fig. 6A); especially the butanol. *T. delbrueckii* also produced a higher concentration of butanol. *St. bacillaris* pure culture fermentation produced the least amount of higher alcohols, partly because not all of the higher alcohols found in the control were detected (Table 11). However, this culture produced the highest concentration of isobutanol compared to the other pure cultures. No butanol was detected. The isoamyl alcohol concentration was also considerably lower than the other cultures.

L. thermotolerans pure culture fermentation resulted in a higher total ester concentration than the control, but this is only due to the high ethyl acetate production (Table 11). The ester concentration at the end of the *T. delbrueckii* fermentation was lower than the control, with the exception of ethyl acetate. The lower ester concentration was mainly attributed to the fact that

this yeast did not produce all the ethyl esters that were detected in the control fermentation. The resulting ester concentration in the *St. bacillaris* pure culture fermentation is the lowest of all the fermentations, mostly due to the low concentration of ethyl acetate. Furthermore, less of the esters measured could be detected after fermentation with this non-*Saccharomyces* yeast compared to all the other fermentations.

It was observed that the non-*Saccharomyces* yeasts produced less volatile acids than *S. cerevisiae* (Table 11). However, *T. delbrueckii* and *St. bacillaris* produced more isobutyric acid than the control (Fig. 7C). Concerning the medium chain fatty acids, *S. cerevisiae* produced considerably higher concentrations than the others.

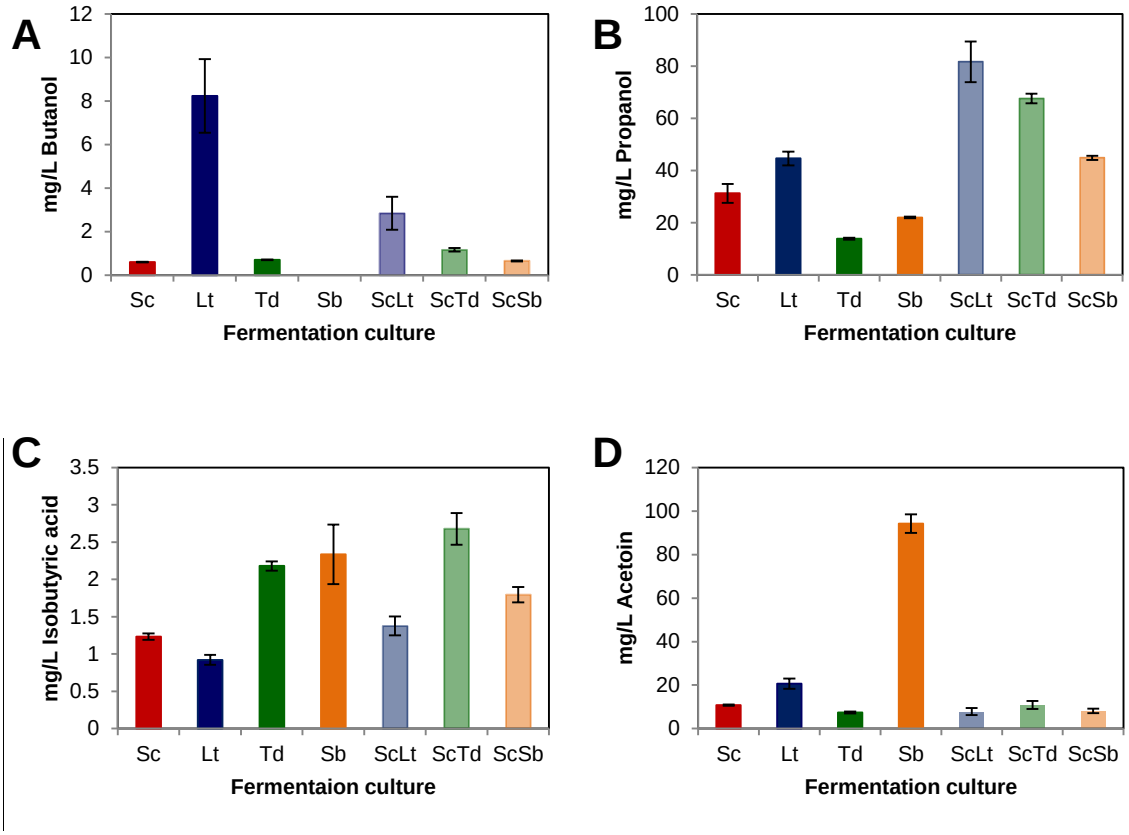

Fig. 6 Concentrations of aroma compounds of interest. A: Butanol; B: Propanol; C: Isobutyric acid; D: Acetoin. Sc: *S. cerevisiae*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sb: *St. bacillaris.*

L. thermotolerans and *St. bacillaris* have a final acetoin concentration that is higher than that of *S. cerevisiae*. In the case of *St. bacillaris* the acetoin concentration is 8.45 folds higher, which is rather significant (Fig. 6D). In contrast, fermentation with *T. delbrueckii* resulted in a lower concentration of acetoin compared to the control.

When the non-*Saccharomyces* yeasts were inoculated together with *S. cerevisiae*, it clearly affected the end concentrations of the volatile aroma compounds compared to the pure cultures. In the mixed cultures, the total higher alcohol levels were noticeably higher than in the control (Table 11). It seems that the high production of propanol and butanol in the

L. thermotolerans pure culture fermentation is observed in the mixed culture fermentation as well (Fig. 6A, B). The higher ester concentration of the mixed cultures in comparison to the control is due to the ethyl acetate concentration. Concerning the organic acids, the mixed culture fermentations resulted in a higher total organic acid concentration than the control; the *S. cerevisiae*-*T. delbrueckii* fermentation showing the highest amount. The higher total is mostly due to isobutyric and propionic acid concentrations. In contrast, the medium chain fatty acids were lower in the mixed cultures than the control, but higher than the pure non-*Saccharomyces* fermentations. The final acetoin concentration in the mixed cultures with *L. thermotolerans* and *St. bacillaris* are lower than either the yeasts present in the mixed culture (Fig. 6D). For the *T. delbrueckii* mixed culture fermentation, the resulting acetoin level is similar to the control.

3.3.2.4 Principal component analysis

Fig. 8 represents the overall chemical outcomes of the fermentations and clear separations can be seen between the pure and mixed culture fermentation and also between *S. cerevisiae* and the non-*Saccharomyces* pure fermentations. The replicates of each fermentation group together, indicating that the fermentation and analytical experimental procedures are reproducible.

Fig. 8A and B show separation based on residual sugar concentrations and primary metabolites including polyols. *St. bacillaris* does not group with any of the other cultures because of its high residual glucose and glycerol concentrations. *S. cerevisiae* pure culture and the mixed cultures separate from the non-*Saccharomyces* pure cultures because of their low residual sugar and high ethanol concentrations. *L. thermotolerans* and *T. delbrueckii* group together because of their similar end point chemical data. However, they do separate somewhat due to higher sorbitol+xylitol production by *T. delbrueckii.*

The PCA plots with the aroma compounds (Fig. 8C, D) also show separation of the non-*Saccharomyces* from *S. cerevisiae* and the mixed cultures. However, in this case, *S. cerevisiae* separates from the other cultures, because of the ethyl ester and medium chain fatty acid concentrations. The increased higher alcohol concentrations of the mixed cultures are apparent, where they group together. In the non-*Saccharomyces* group, acetoin and butanol pull *St. bacillaris* and *L. thermotolerans* apart, respectively, from the rest of the group.

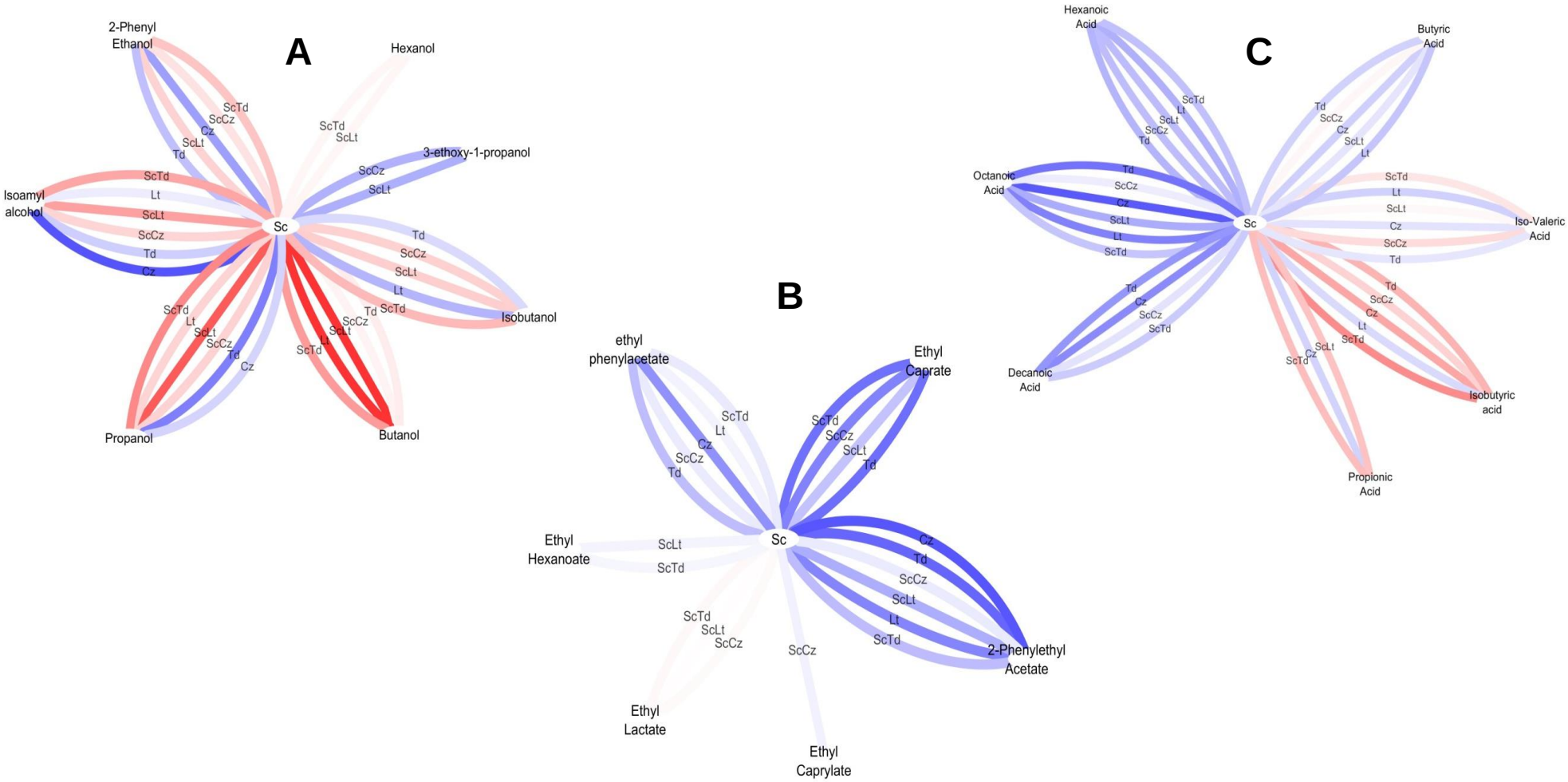

Fig. 7 Network diagrams showing how the pure and mixed culture fermentations differ from *S. cerevisiae*. Only compounds and fermentations that differ significantly are shown. The colour of the edges indicates if a fermentation culture produced more (red) or less (blue) of a certain compound. The intensity of the edge indicates how much difference from the control, brighter, more of a difference.

A: Higher alcohols; B; Esters; C: Fatty acids. Sc: *S. cerevisiae*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sb: *St. bacillaris.*

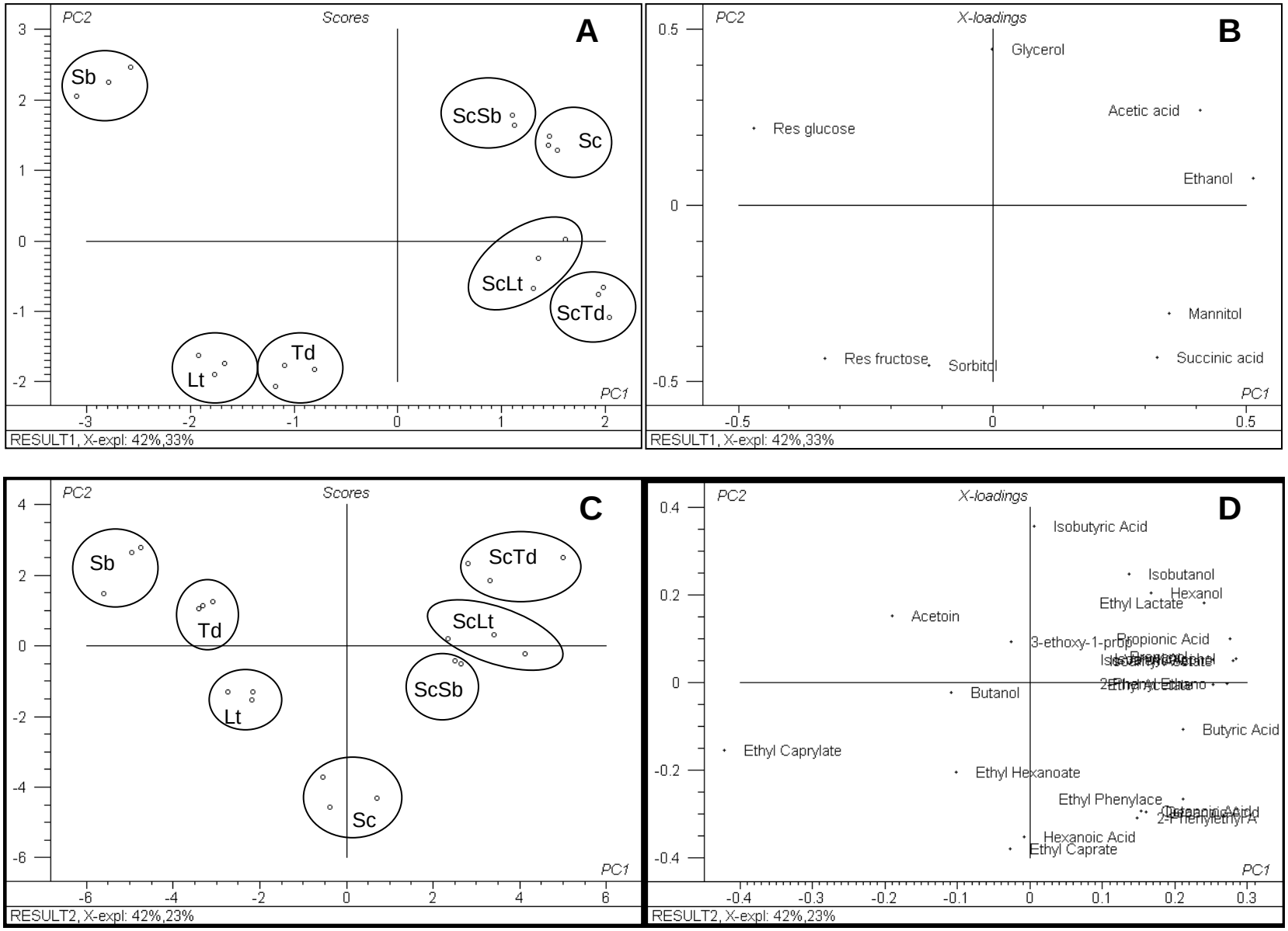

Fig. 8 PCA plots showing separation of different fermentation cultures in terms of end of fermentation concentrations of compounds. A,B: Scores and loadings plots based on concentrations of residual sugars and primary metabolites; C,D: Scores and loadings plots based on concentrations of volatile aroma compounds. Sc: *S. cerevisiae*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*; Sb: *St. bacillaris.*

3.3.3 Sequencing of selected genes (*ALD6*, *GPD1*, *GPD2*, *GPP1* and *GPP2*) in *L. thermotolerans* and *T. delbrueckii*

The data suggest a significant difference between the investigated species with regards to the regulation of glycerol and acetic acid metabolism. In order to investigate whether these differences might be related to core glycerol and acetic acid metabolic pathway genes, the corresponding genes were cloned and sequenced to design species-specific primers for RT-qPCR (Refer to the materials and methods section).

It was observed that all the genes sequenced are homologous between the species (*S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii*) (Fig. 9). The similarity scores of the nucleotide and amino acid sequence alignments are between 64% and 84% (data not shown). Furthermore, *T. delbrueckii* is more similar to *S. cerevisiae* than *L. thermotolerans* is for these sequences (with the exception of *ALD6* amino acid sequence). It was also found that the two non-*Saccharomyces* yeasts do not seem to possess a homolog to the *GPD2* gene of *S. cerevisiae*. The *S. cerevisiae GPD1* and *GPD2* genes are clearly different from each other, with a similarity score of 67%. The non-*Saccharomyces*' *GPD1* is more similar to *S. cerevisiae*'s *GPD1* than to its *GPD2*. The *GPP1* genes of the two non-*Saccharomyces* species are more similar to *S. cerevisiae*'s *GPP2*. Like the gene families sequenced and compared above, the *ALD6* sequences of the different species are similar. However, when the amino acid sequences were aligned, a nine amino acid gap was observed for the *T. delbrueckii* strain used in this study. Furthermore, the gap was also noticed in the alignment with the sequenced *T. delbrueckii* genome (strain CBS 1146).

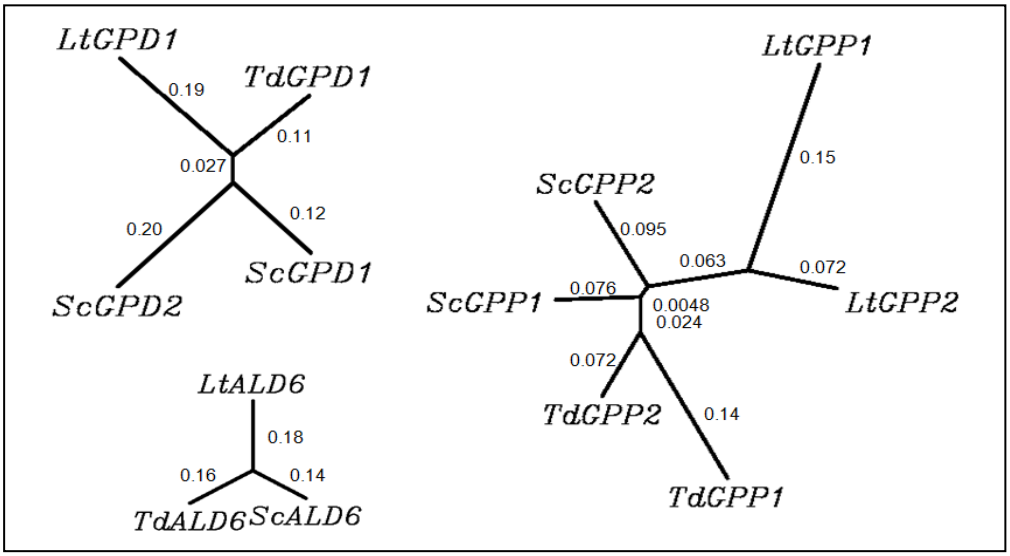

Fig. 9 Unrooted phylogenetic tree (neighbour joining) with branch lengths showing the relatedness of the genes sequenced in this study. The branch lengths are based on the similarity scores of the nucleotide sequences (Clustalw).

Species specific primers for RT-qPCR were designed in variable regions of the gene sequences mentioned above. The designed primers were tested on the RT-qPCR machine to determine the PCR efficiencies and the specificity of the primers (Table 12). PCR efficiencies are considered acceptable between 90 and 110% (Life Technologies Corporation 2011). The PCR reactions carried out with the designed primers were therefore regarded as efficient (Table 12). However, it was observed that the primers were not optimally species specific. The primer pairs of each species indeed amplified the DNA of all three species, although with a lower efficiency for the species that the primers were not supposed to amplify. This observation makes it difficult to distinguish between gene expression of different species in a mixed culture. For this reason, we did not attempt determining the gene expression in the mixed cultures.

Table 12 PCR efficiencies of the primers designed for RT-qPCR

Name	PCR efficiency (%)	R^2
ScGPD1qPCRfw / **ScGPD1qPCRrv**	98.92	0.999
LtGPD1qPCRfw / **LtGPD1qPCRrv**	89.34	1
TdGPD1qPCRfw / **TdGPD1qPCRrv**	88.02	0.998
ScGPD2qPCRfw / **ScGPD2qPCRrv**	97.09	0.996
ScALD6qPCR2fw / **ScALD6qPCR2rv**	96.65	0.999
LtALD6qPCR2fw / **LtALD6qPCRrv**	110.59	0.995
TdALD6qPCRfw / **TdALD6qPCRrv**	99.84	0.999

Sc: *S. cerevisiae*; Lt: *L. thermotolerans*; Td: *T. delbrueckii*.

However, an attempt was made to determine the gene expression of *GPD1* and *ALD6* in the pure culture fermentations. Total RNA was extracted from three different time points in the fermentation and cDNA was synthesized. The attempt was unsuccessful due to seemingly too little cDNA. The amplification was only visible after 35 or more cycles of amplification, and for some of the samples the amplification profile could not be distinguished from the negative control, with water as the template.

3.4 Discussion

The aim of the present study was to investigate whether the non-*Saccharomyces* yeasts *L. thermotolerans*, *T. delbrueckii* and *St. bacillaris* behave differently from *S. cerevisiae* in the

initial hyperosmotic stress when inoculated into grape must for alcoholic fermentation. We focussed on the production of certain metabolites, in particular those produced (at least in part) as a direct or indirect response to osmotic stress, in fermentations performed in a synthetic grape must medium. Furthermore, each non-*Saccharomyces* yeast was inoculated with *S. cerevisiae* to investigate the yeast-yeast interactions and consequently their effect on wine composition. Differences in fermentation behaviour and metabolite production were observed between all yeast species. Furthermore, mixed culture fermentations behaved differently than the individual pure cultures in terms of metabolites produced and sugar consumption rate.

3.4.1 Fermentation behaviour

The non-*Saccharomyces* pure cultures fermented slower than *S. cerevisiae* pure culture fermentation (the control) and got stuck with high residual sugar concentrations. However, their ethanol yields were similar to that of the control. Non-*Saccharomyces* yeasts, including the species used in this study, are known to have lower fermentation abilities than *S. cerevisiae* (Ciani and Maccarelli 1998, Jolly et al. 2006, Ciani et al. 2010, Comitini et al. 2011). The mixed culture fermentations fermented to dryness although at a slower rate than *S. cerevisiae*. Our results confirm the available literature; non-*Saccharomyces* yeasts rarely persist until the end of fermentation when *S. cerevisiae* is present (Nissen and Arneborg 2003, Ciani et al. 2006, Comitini et al. 2011). In contrast, *S. cerevisiae* remained at high cell concentrations until fermentation completion. Therefore, it is most likely *S. cerevisiae* that completed the fermentations and the non-*Saccharomyces* yeasts that led to the slower fermentation rate. Clearly, in the mixed culture fermentation, the yeasts interact with each other. Our results suggest that the interactions led to the early death of the non-*Saccharomyces* yeasts. Factors that may contribute to the decline in cell count in mixed cultures include increasing ethanol concentration, toxic medium chain fatty acids, nutrient deficiencies or inhibitory peptides (Alexandre et al. 1998, Fleet 2003) as well as oxygen availability, space limitation and cell-to-cell contact (Hansen et al. 2001, Nissen and Arneborg 2003).

The ethanol concentrations were measured at the point where the non-*Saccharomyces* yeasts started to die off in the mixed cultures and compared to their tolerances found in previous studies. The different non-*Saccharomyces* concentrations started to decrease at ethanol levels between 9 and 10% v/v in the mixed cultures. Pure cultures of these yeasts reached final ethanol concentrations of 8-10% v/v. Therefore, it is unlikely that the ethanol concentration was the main reason for these yeasts to die off in the mixed culture. Furthermore, it has been reported that *L. thermotolerans*, *T. delbrueckii* and *St. bacillaris* can survive at ethanol levels up to 9, 16.5 and 14% v/v respectively, although it is greatly strain and condition dependent (Sipiczki 2004, Kapsopoulou et al. 2005, Cordero-Bueso et al. 2010, Azzolini et al. 2012, Tofalo et al. 2012).

The resulting ethanol concentrations of the mixed culture fermentations were one or two percent lower than the control. It would prove useful to confirm the lower ethanol concentrations, since biological methods to lower ethanol in wines are currently sought (Gobbi et al. 2014). The use of non-*Saccharomyces* yeasts in mixed cultures with *S. cerevisiae* has been suggested for this purpose (Bely et al. 2013, Gobbi et al. 2013, Contreras et al. 2014) and our results would confirm their suitability to achieve this purpose.

3.4.2 Polyol and acetic acid production

Yeasts produce polyols as osmoprotectants in a high solute concentration medium such as grape must (van Eck et al. 1993). In the present study, the extracellular glycerol concentrations were determined throughout the fermentations. It was observed that *L. thermotolerans*, *T. delbrueckii* and *St. bacillaris* produced lower, similar and higher amounts of glycerol than the control, respectively. *St. bacillaris* is known to produce high levels of glycerol (Soden et al. 2000, Sipiczki 2004, Tofalo et al. 2012). However, lower levels of glycerol than *S. cerevisiae* have been reported for *L. thermotolerans* and *T. delbrueckii* (Ciani and Maccarelli 1998, Kapsopoulou et al. 2005, Renault et al. 2009). Therefore, the similar amounts that *T. delbrueckii* produced could be due to strain differences as reported in literature (Ciani and Maccarelli 1998, Renault et al. 2009).

Although glycerol is the main compatible solute produced by yeast, other polyols can also be produced (e.g. sorbitol, xylitol, mannitol and arabitol) (Tokuoka et al. 1992). Therefore, in this study, the production of sorbitol+xylitol and that of mannitol+arabitol were determined and compared to the control. Sorbitol and xylitol were measured together, since the enzyme kit used cannot distinguish between these two polyols; the same applied for mannitol+arabitol. It was observed that *L. thermotolerans* and *T. delbrueckii* produced increased amounts of these polyols compared to *S. cerevisiae*. *St. bacillaris* produced less mannitol+arabitol, but more sorbitol+xylitol. Because this yeast produced high levels of glycerol, it probably did not require the production of additional polyols for osmoregulation. In literature, the production of other polyols than glycerol has not been investigated in wine fermentations. Furthermore, the reason for producing other polyols as compatible solutes together with glycerol is not known. Davis et al. (2000) observed that at physiological concentrations the polyols with four to six carbon atoms did not yield differences in terms of osmotic balance. Therefore, the reason is not the osmoprotective ability of a particular polyol.

Glycerol is not only produced as compatible solute, but also synthesized to maintain intracellular redox balance under anaerobic conditions. Glycerol production oxidizes the NADH surplus formed during glycolysis for biomass production (Norbeck et al. 1996, Bakker et al. 2001). Consequently, during fermentation in a medium such as grape must, this polyol is produced in high amounts and could cause an intracellular redox imbalance that leads to the production of other by-products such as acetic acid (Bakker et al. 2001). The production of

glycerol is therefore concomitant with acetic acid production in *S. cerevisiae* (Remize et al. 1999). This phenomenon has not been reported for non-*Saccharomyces* yeasts. In this study, the acetic acid concentrations were determined throughout fermentation. We observed that the non-*Saccharomyces* yeasts produced less acetic acid than *S. cerevisiae*, even *St. bacillaris*, which produced high glycerol concentrations. Furthermore, the non-*Saccharomyces* yeasts rather produced acetic acid gradually throughout fermentation in contrast with *S. cerevisiae* that produced the majority of acetic acid in the beginning of fermentation, during the biomass and glycerol production phase as previously reported (de Barros Lopes et al. 2000, Remize et al. 2001). It has been shown that *L. thermotolerans*, *T. delbrueckii* and *St. bacillaris* are constant producers of low levels of acetic acid (Ciani and Maccarelli 1998, Kapsopoulou et al. 2005, Renault et al. 2009, Ciani and Ferraro 1998, Magyar and Toth 2011, Tofalo et al. 2012). However, this characteristic is strain dependent in the case of *T. delbrueckii* and *St. bacillaris* (Renault et al. 2009, Soden et al. 2000, Sadoudi et al. 2012). Furthermore, in this study, the acetic acid yields per gram of sugar consumed were higher for *T. delbrueckii* and *St. bacillaris* compared to the control. Therefore, if these yeasts were to complete fermentation on their own, the acetic acid concentrations could be higher than that of *S. cerevisiae*.

When two yeasts are inoculated together for alcoholic fermentation, they interact and these interactions affect the final polyol and acetic acid concentrations. In this study, it was found that the glycerol concentrations are higher in the mixed cultures than *S. cerevisiae* pure culture. It confirms what is reported in literature (Ciani and Ferraro 1998, Kapsopoulou et al. 2007, Bely et al. 2008, Azzolini et al. 2012, Comitini et al. 2011, Gobbi et al. 2013). The increased glycerol levels are probably due to a cumulative effect of the two yeasts present. For the *St. bacillaris* mixed culture, the final glycerol concentration was lower than for the *St. bacillaris* pure culture, which is a consequence of the dominance of *S. cerevisiae.* Concerning the other polyols, the mixed cultures produced more than *S. cerevisiae.* Therefore, it is mostly the contribution of the non-*Saccharomyces* yeast in the mixed culture. As far as we know, no literature on polyols other than glycerol in wine yeasts and mixed cultures is available to confirm or contradict our results. Concerning the effect on acetic acid concentrations, it was observed that the mixed culture fermentations resulted in less acetic acid than *S. cerevisiae.* How the two yeasts present in the mixed culture fermentation interact to lower the acetic acid is unclear. The mixed culture with *L. thermotolerans* resulted in low acetic acid concentration while the non-*Saccharomyces* yeast was present in high numbers. *L. thermotolerans* produces low levels on its own, but it is possible that it consumes the acetic acid produced by *S. cerevisiae* as well. The consumption of acetic acid together with glucose in limited-aerobic conditions has indeed been reported for *L. thermotolerans* and *Zygosaccharomyces bailii* (Vilela-Moura et al. 2008, Rodrigues et al. 2012). With regards to *T. delbrueckii*, acetic acid uptake and metabolism is repressed in the presence of glucose, as for *S. cerevisiae* (Casal and Leao 1995, dos Santos et al. 2003). Therefore, the consumption of acetic acid is not a likely cause for decreased acetic

acid levels in this mixed culture. A hypothesis is that yeasts like *T. delbrueckii* alleviate the stress by utilizing some of the sugars in the beginning of fermentation before *S. cerevisiae* is inoculated, thereby preventing *S. cerevisiae* from producing acetic acid (Rantsiou et al. 2012).

3.4.3 Volatile aroma compound production

Since the non-*Saccharomyces* yeasts produced different levels of glycerol, various polyols and acetic acid than *S. cerevisiae*, they might have another way to maintain redox balance in the initial stages of fermentation. In order to test this hypothesis, we measured various higher alcohols, esters and volatile acids that incidentally have an impact on wine aroma.

Higher alcohols are mostly produced through the Ehrlich pathway where the yeast can synthesize either a higher alcohol or an acid from the corresponding amino acids. This depends on the redox status of the cell. If the higher alcohol is produced, NADH is released and if the corresponding acid is produced, NAD^+ is released. Overall, the non-*Saccharomyces* yeasts produced lower total higher alcohol concentrations than *S. cerevisiae*. However, *L. thermotolerans* and *T. delbrueckii* produced more butanol and the former also produced increased levels of propanol. The high concentrations of butanol and propanol by *L. thermotolerans* have previously been reported in literature (Gobbi et al. 2013, Mains 2014). It may well be that these non-*Saccharomyces* yeasts produced the higher alcohols in order to maintain redox balance in anaerobic conditions (Hazelwood et al. 2008) as *S. cerevisiae* does with glycerol production. Furthermore, higher alcohols contribute to the wine aroma. Below 300 mg/L, these compounds can add to the desirable complexity of the wine, but above 400 mg/L, it could be detrimental to wine quality (Swiegers and Pretorius 2005). All the fermentations performed in this study resulted in lower than 300 mg/L higher alcohols. As for the increased levels of butanol and propanol from *L. thermotolerans*, it is below the odour thresholds of these compounds in wine (Peinado et al. 2004, Escudero et al. 2007). Overall, *T. delbrueckii* and *St. bacillaris* produced higher concentrations of volatile acids than the control, mostly due to the isobutyric acid concentration. Andorrà et al. (2010) indeed reported high levels of isobutyric acid for *St. bacillaris* in comparison to *S. cerevisiae*. *L. thermotolerans* produced higher levels of butanol and lower levels of butyric acid than the control. Therefore, it could be that this yeast rather produced the alcohol than the acid in the Ehrlich pathway, possibly for redox balance; NAD^+ rather than NADH was required.

Succinic acid is produced through the reductive branch of the TCA cycle in fermentative conditions and it is proposed that its production participates in the maintenance of the redox balance during fermentation by regenerating FAD, rather than having an impact on the NADH (Camarasa et al. 2007). It has been previously observed that *T. delbrueckii* produces significant amounts of this acid (Ciani and Maccarelli 1997) and it was confirmed in this study. Conversely, we observed a low production of succinic acid for *St. bacillaris* when compared with *S. cerevisiae*; the same was reported by Magyar et al. (2014). However, there are reports of this

yeast producing high levels of this particular acid as well (Ciani and Ferraro 1998, Andorrà et al. 2012).

Concerning acetoin, *St. bacillaris* produced the highest concentration. It confirms what is reported in literature (Romano and Suzzi 1996, Ciani et al. 2000). In contrast, *T. delbrueckii* produced less acetoin than the control, confirming what was found by Ciani et al. (2006). Furthermore, the latter authors reported that *L. thermotolerans* produced low amounts of acetoin, although in this study it produced double the concentration of *S. cerevisiae*. This might be a strain dependent characteristic. It is likely that acetoin is produced to maintain redox balance, by regenerating NAD^+. Furthermore, it could be a way to rid the cell of toxic amounts of acetaldehyde (Remize et al. 1999). In *St. bacillaris*, acetoin might be accumulated because there are not enough NADH to reduce acetoin to 2,3-butanediol, since there is a high production of glycerol.

The formation of esters is not directly correlated with redox balance, but the production of their precursors is, as mentioned above. It was found that the non-*Saccharomyces* yeasts produced less esters in total than *S. cerevisiae;* this is confirmed in literature (Sadoudi et al. 2012, Gobbi et al. 2013). However, ester production varies depending on species and strains (Rojas et al. 2001, Viana et al. 2008). Furthermore, Andorra et al. (2010) found that *St. bacillaris* produced more esters than *S. cerevisiae*. In addition, Gobbi et al. (2013) reported that *L. thermotolerans* produced lower ester concentrations in total, but with higher levels of certain individual esters.

In the mixed culture fermentations, the interactions between the two yeasts also lead to differences in aroma compound concentrations. However, the kind of interaction occurring between the yeasts is not clear. In this study, it was observed that the mixed culture fermentations resulted in increased total concentrations of higher alcohols, esters and short chain volatile acids compared to the control. The higher concentrations are most likely a cumulative effect of the two yeasts present. In literature, it is shown that mixed cultures with non-*Saccharomyces* are beneficial for wine aroma (Jolly 2006, Ciani et al. 2010, Azzolini et al. 2012, Gobbi et al. 2013). Regarding the medium chain fatty acids, *S. cerevisiae* produced more than all the other cultures. Interestingly, in the mixed culture of *St. bacillaris* with *S. cerevisiae*, the levels of acetoin were lower than either pure culture. The same is observed for the *L. thermotolerans*-*S. cerevisiae* fermentation. It could be that *S. cerevisiae* takes up the acetoin and converts it to 2,3-butanediol as suggested by Ciani and Ferraro (1998).

3.4.4 Gene sequences and expression

Investigating non-*Saccharomyces* yeasts in wine fermentation has so far been impaired due to a lack of genetic information; most of the genomes are indeed not yet known. However, the genomes of *L. thermotolerans* and *T. delbrueckii* were recently sequenced, although not fully annotated. In this study, we aimed to evaluate how these yeasts interact with *S. cerevisiae* in

mixed culture fermentations in terms of gene expression. Selected genes (*GPD1*, *GPD2*, *GPP1*, *GPP2* and *ALD6*) involved in glycerol and acetic acid production were sequenced. Subsequently, the nucleotide and amino acid sequences were compared to identify intraspecific variations.

It was observed that the genes sequenced are fairly homologous between species. However, a nine-amino acid (27-nucleotide) gap in the *T. delbrueckii* CRBO L0544 strain for *ALD6* in comparison to the sequenced strain (CBS 1146) and to the other species was observed. The gap is in the 230-238 amino acid position and in the *S. cerevisiae* sequence, amino acids at position 233 and 234 constitute a NAD(P)-binding site and the deletion could therefore impact on the enzyme activity. However, this is one of several NAD(P)-binding sites and further investigation is needed to determine whether this site is of importance in acetic acid production. Furthermore, the two non-*Saccharomyces* yeasts do not seem to possess a homolog to the *GPD2* gene of *S. cerevisiae*. This gene is responsible for glycerol formation in oxygen limiting conditions (Remize et al. 2001). It has been shown that the non-*Saccharomyces* yeasts are less tolerant to low oxygen concentrations than *S. cerevisiae* (Hansen et al. 2001). Thus, the absence of a *GPD2* gene might contribute to this phenotype. However, there are reports of a *GPD2* homologue in other non-*Saccharomyces* yeasts, such as *Zygosaccharomyces rouxii* (Iwaki et al. 2001), *Schizosaccharomyces pombe* (Ohmiya et al. 1995), *Candida albicans* (Enjalbert et al. 2003) and *Pichia jadinii* (Osterman et al. 2006). Therefore, the seeming absence of this gene and its biological consequences require further investigation.

The rationale for sequencing the selected genes was to obtain the exact sequences for the strains used in this study in order for primers to be designed for RT-qPCR. Following sequence alignment, primers were designed in variable regions of the genes, because it is crucial to distinguish between expression of *S. cerevisiae* and the non-*Saccharomyces* yeast in a mixed culture. The designed primers were tested on genomic DNA and the PCR efficiencies and the specificity of the primers determined. However, it was observed that the primers were not optimally species specific. We proceeded to only determine gene expression of the pure cultures.

RNA was extracted on days 2, 7 and 12 of all the fermentations and reverse transcribed to cDNA. The samples were subjected to RT-qPCR with the species-specific *GPD1* and *ALD6* primers, but the attempt at measuring gene expression failed. The reason was most likely due to too little cDNA present in the reaction. The problem was not with the reverse transcription reaction, as the positive control yielded a satisfactory result. We theorized that the storage of the cell pellets before RNA extraction in terms of time and temperature could be a major factor. Indeed, when RNA was extracted from an overnight culture, the RNA yield was high and accordingly the cDNA concentration. This finding can point to the storage of the cell pellets, or alternatively to the growth conditions. The overnight culture was grown in YPD at 30°C with

agitation; these are optimal growth conditions. However, the fermentation cultures were in a stressful environment for a few days. This might strengthen the cell walls, presenting a challenge in breaking the cells to release the RNA.

Due to a lack of samples and time, the above theories could not be fully tested or gene expression experiments repeated. Furthermore, in literature, gene expression studies on *GPD1* and *ALD6* in fermenting yeast were carried out on samples taken in the first few hours up to three days of fermentation (Remize et al. 2003, Sadoudi et al. 2014). This is reasonable, since the osmotic stress response is activated and accomplished within a few hours (Hohmann 2002). Therefore, if these gene expression experiments were to be repeated, it should be carried out on samples taken early in the fermentations.

3.4.5 Conclusion

It was found that the non-*Saccharomyces* yeasts used in this study produce other polyols in addition to glycerol, most likely to counteract the osmotic stress due to the high initial sugar concentrations of grape must. However, the polyols are not produced in such high concentrations comparable to glycerol. Glycerol is also produced in *S. cerevisiae* to maintain redox balance in anaerobic conditions, higher alcohols may also serve as redox sinks. We observed elevated levels of butanol and propanol from *L. thermotolerans.* Furthermore, we confirmed the low production of acetic acid from the non-*Saccharomyces* yeasts. Therefore either these yeasts do not have a need for NADH or they produce other compounds to maintain redox balance when there is an increase in glycerol production. In this study, elevated levels of isobutyric acid were observed for *T. delbrueckii* and *St. bacillaris*, but further research needs to be conducted.

Although the non-*Saccharomyces* yeasts have lower fermentation abilities than *S. cerevisiae*, they are good candidates for fermentation together with *S. cerevisiae* since they lower the acetic acid concentration and contribute positively to the wine aroma. Furthermore, the mixed culture fermentations showed the potential of lowering the ethanol concentrations of wine.

From the gene sequencing data, we found that these particular genes are rather conserved, but there are interesting differences that could be followed up on in order to broaden our knowledge on the non-*Saccharomyces* yeast on a molecular level. Furthermore, it would be valuable to repeat the expression experiments to investigate whether one yeast in a mixed culture fermentation affect the gene expression profile of the other.

3.5 References

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Chapter 4

General discussion and conclusions

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4.1 General discussion and conclusions

Redox balancing and osmotic stress adaptation are essential cellular functions. In the case of yeasts, most data related to such processes have been generated using the model species *Saccharomyces cerevisiae* (Nevoight and Stahl 1997, Rep et al. 2000, Hohmann 2002). These data have contributed significantly to our broader understanding of such vital processes. However, and as the data of this study confirm, it is clear that even taxonomically closely related species appear to have evolved significantly different metabolic stress response pathways.

In this study, we aimed to investigate the metabolic response to hyper-osmotic stress and redox imbalance during wine fermentation. Our data confirm that the non-*Saccharomyces* yeasts produce other polyols in addition to glycerol most likely in response to the high sugar concentration of the fermentation medium. Certain non-*Saccharomyces* yeasts have indeed been shown to produce other polyols as well in response to osmotic stress as summarized in the literature review of this thesis (Tokuoka et al. 1992, van Eck et al. 1993).

Furthermore, glycerol is produced to maintain redox balance during fermentative conditions, to oxidise the surplus NADH generated in biomass formation (Bakker et al. 2001). It has been proposed that higher alcohols can fulfil this function as well (Schoondermark-Stolk et al. 2005, Hazelwood et al. 2008). Our results showed that fermentation with *Lachancea thermotolerans* resulted in significant concentrations of butanol and propanol. The lower glycerol levels of *L. thermotolerans* compared to *S. cerevisiae* could suggest that the cells were in need for NAD^+. However, it is only an assumption and should be systematically investigated.

Another difference between *S. cerevisiae* and non-*Saccharomyces* wine yeasts that has been reported in literature, is the concomitant production of acetic acid with glycerol in *S. cerevisiae* when the yeast is in grape must (Remize et al. 1999, Erasmus et al. 2004). The results obtained in this study show low resulting acetic acid concentrations in the non-*Saccharomyces* fermentations. Furthermore, in the case of *Starmerella bacillaris*, it is evident that the acetic acid production is not a consequence of high glycerol production. It might be that *S. cerevisiae* produces the acetic acid to reduce NAD^+ generated by a high production of glycerol. We observed an increase in isobutyric acid in fermentations with *Torulaspora delbrueckii* and *St. bacillaris* in comparison to *S. cerevisiae.* This acid might well be produced when the cell requires NADH. It should be kept in mind that we investigated and discussed the production of only a few metabolites in isolation and redox balance governs most metabolic reactions. Therefore, the results obtained may only be interpreted with caution and should be used as a basis for further research on the topic.

Non-*Saccharomyces* yeasts can be beneficial in wine fermentations, but due to low fermentation abilities research into mixed cultures with *S. cerevisiae* have been conducted (Ciani et al. 2006, Comitini et al. 2011). In this study, the outcome of mixed culture fermentations on metabolite level was also assessed. The results confirm the benefit of using these non-*Saccharomyces* yeasts. The higher glycerol concentrations can contribute to a smoother mouthfeel in the wine and the lower acetic acid leads to a reduction in volatile acidity thereby enhancing the quality of the resulting wine. Furthermore, we observed lower ethanol concentrations, a characteristic that is sought by many wine consumers.

Finally, the data in this study definitely suggest that the non-*Saccharomyces* yeasts behave differently in response to certain environmental conditions than *S. cerevisiae*. The extent of these differences needs to be further explored in order not only to improve our general knowledge of these yeasts, but also to utilize their beneficial characteristics in wine making. The more is known about these yeasts the more they can be exploited to our benefit.

4.2 Potential future research

A more extensive study into the mechanism of osmoregulation in wine-related non-*Saccharomyces* yeasts could provide further insight into how some of these yeasts survive on ripe grape berries and in grape must fermentation. Therefore, to fully assess the effect of hyper-osmotic stress on the fermenting yeasts and the resulting polyol concentrations, fermentation in very high sugar (300 - 400 mg/L) medium could be performed. Furthermore, the production of polyols could be measured throughout fermentation and the production trend compared to that of glycerol. In order to determine individual polyol concentrations, a more sensitive method should be applied, such as GC-MS.

The metabolic impact of the non-*Saccharomyces* yeasts in the mixed cultures could be further assessed by investigating different inoculation strategies regarding ratios of cell concentrations and timing of sequential inoculations. In order to establish how the non-*Saccharomyces* yeasts (especially *L. thermotolerans*) lower the acetic acid in the mixed culture fermentations, an experiment could be carried out to evaluate whether the non-*Saccharomyces* yeasts can consume acetic acid together with glucose.

Although this study provides some insight into the metabolic behaviour of non-*Saccharomyces* yeasts in fermentation, it was performed in synthetic grape must. Therefore, the fermentations need to be repeated in real grape must in order to confirm the results. Furthermore, the contribution of the metabolites produced to the wine aroma and sensorial characteristics need to be intensively studied, as not all strains are equally beneficial for wine quality. Additionally, the ability of the presence of the non-*Saccharomyces* yeasts in the mixed culture to lower the ethanol levels could be confirmed in the real grape must.

The gene expression experiments could be repeated from samples taken earlier in the fermentation, in order to determine the gene expression of *GPD1* and *ALD6* in the mixed culture

fermentations. This would allow assessing whether the yeasts affect one another on a genetic level. Subsequently, the activities of the enzymes encoded by these genes could be evaluated and correlated with gene expression and metabolite production.

Ultimately, the goal should be to find out more about these non-*Saccharomyces* yeasts, their cellular responses in fermentations and consequently the impact on wine composition and organoleptic properties. This could make it possible for these yeasts to be better exploited in wine making leading to different styles and better quality wines.

4.3 References

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